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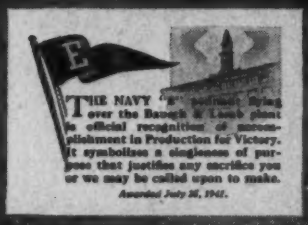
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THE AMERICAN JOURNAL OF PATHOLOGY

VOLUME XIX MARCH, 1943 NUMBER 2

CALCIFICATION AND PHOSPHATASE *

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In 1923, Robison made the observation that if calcium or barium salts of hexosephosphate are incubated with an extract of bone containing phosphatase, a precipitate of calcium or barium phosphate, respectively, will form. This phenomenon led him to the idea that phosphatase may play a rôle in calcification of bone as it occurs *in vivo*. One year later he found that phosphatase is also present in developing teeth of young animals. Further studies by Martland and Robison showed that phosphatase is not present in cartilage before the appearance of the center of ossification but can be demonstrated in large amounts after the appearance of the center. Fell and Robison cultured embryonic fowl femora *in vitro* and found that the appearance of phosphatase coincides in time with the development of hypertrophied cartilage. Huggins showed that considerable amounts of phosphatase are present in and around bone-producing transplants of bladder mucosa. This finding was confirmed by Regen and Wilkins. The findings mentioned, together with the well known changes in plasma phosphatase level in diseases of the bones (Kay), support the assumption of a close causal relationship between calcification and the presence of phosphatase. Although it has been known since the experiments of Shipley that *in vitro* calcification of calcifiable tissues will occur even in purely inorganic solutions, apparently without phosphatase action, the results of recent studies by Gutman and Gutman indicate that even in the case of inorganic solutions phosphatase plays an important rôle by breaking down hexosephosphoric esters formed locally from glycogen and inorganic phosphate. In conclusion, it seems that phosphatase action is at least one of the mechanisms utilized by the organism to increase the $[Ca^{++}] \times [PO_4^{\equiv}]$ product locally beyond the critical value of 3.3×10^{-6}, which is the solubility product of secondary calcium phosphate at the pH of the blood.

* Aided by grants from the Douglas Smith Foundation for Medical Research of the University of Chicago and from the Committee on Scientific Research of the American Medical Association.

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In 1939 a microtechnical method for the visualization of phosphatase action in tissue sections (Gomori, Takamatsu) became available. Shortly afterward, Ross, and Freeman and McLean published morphological observations on the presence of phosphatase in calcifying trichina cysts and in bone. So far no other histological observations on the relationship between calcification and phosphatase have been published.

In the present paper the relationship between phosphatase and calcification under normal and under a variety of pathological conditions, as shown by a new microtechnical method for the simultaneous visualization of preformed calcium salt deposits and of phosphatase activity, is presented.

Material and Methods

Since calcium salts are removed and phosphatase destroyed by decalcification, all material was embedded and cut without decalcification. This imposed a certain limitation on the material since tissues too hard to be sectioned without decalcification had to be excluded.

Slices of tissues were fixed in 80 per cent alcohol not later than 4 hours after death or removal at operation. Small embryos were fixed *in toto*. The tissues were subsequently dehydrated in 95 per cent and absolute alcohol and embedded in paraffin. Some of the tissues were fixed in ice-cold acetone for studies on acid phosphatase. Paraffin sections were cut from 4 to 6 μ in thickness.

The sections were stained with a modification of my method for the demonstration of phosphatase, devised for the purpose of simultaneous visualization of preformed deposits of calcium salts and of sites of phosphatase activity. The principle of the modification is this: insoluble calcium salts are first demonstrated by transforming them into black cobalt sulfide. The section is subsequently incubated with a solution of calcium glycerophosphate. The calcium phosphate precipitate formed by enzymatic action is stained in a different shade.

The method is as follows:

1. Fix fresh tissues in 80% alcohol, embed in paraffin.
2. Run paraffin sections through xylol and alcohols to distilled water.
3. Treat sections for from 6 to 12 hours with a 2% solution of cobalt acetate. Calcium phosphate and carbonate are transformed into the corresponding cobalt salts. Rinse sections thoroughly in distilled water.
4. Immerse sections for 10 minutes in dilute buffered solution of yellow ammonium sulfide (5 to 6 drops of yellow ammonium sulfide to a Coplin jarful of phosphate or maleate buffer of about pH 7). Unbuffered, strongly alkaline solutions of ammonium sulfide may destroy the enzyme. Cobalt phosphate and carbonate are transformed into black cobalt sulfide. Rinse in water.

5. Incubate sections for from 5 to 6 hours at 37° C. in the following solution:

2% sodium glycerophosphate	25 cc.
2% sodium barbital	25 cc.
Distilled water	50 cc.
2% calcium chloride	5 cc.
2% magnesium sulfate	2 cc.
Chloroform	a few drops

This solution will keep in the ice box for months. Before use add to this mixture a few drops of a 1% solution of some soluble sulfide, such as ammonium or sodium sulfide, in order to depress the solubility of the cobalt sulfide precipitate.

After incubation rinse sections thoroughly in distilled water.

6. Transfer sections for 15 minutes to a 1% solution of lead nitrate. The calcium phosphate precipitate formed at the sites of phosphatase activity is transformed into lead phosphate. Rinse with distilled water.

7. Stain sections in the following mixture for 15 minutes:

0.5% solution of methyl green	2 to 3 parts
0.5% solution of acridine red (Grübler)	1 part

Other brands of acridine red gave results which were much inferior. The staining solution should be not more than 4 weeks old. It should be kept in the ice box when not in use.

8. Differentiate in 95% alcohol, dehydrate in absolute alcohol, clear in xylol, mount in balsam.

Results: preformed deposits of calcium salts, black; sites of phosphatase activity, purplish red; nuclei, green-blue.

Observations

The normal material consisted of numerous embryos of the mouse, rat, guinea-pig, rabbit, dog, pig, chicken and man. The findings were essentially the same in all species.

The earliest sites of phosphatase-positive staining in the skeletal system were the perichondrium of the vertebrae and of the ribs, but in a somewhat later stage the perichondrium of practically all cartilage that in later life develops into bone became positive. The perichondrium contained phosphatase either all around the circumference of the cartilage or in certain patches only. Somewhat later the cartilage itself, especially the portion adjoining the positively-staining perichondrium, showed an intense reaction both in the cell nuclei and in the matrix. It was in these sites of positive phosphatase reaction within the cartilage that the first traces of granular calcification appeared. The deposits of calcium salts were always found in the centers of phosphatase-positive areas. Such areas were about 20 to 50 μ wide. At practically the same stage or somewhat later, cellular strands of connective tissue, representing the anlage of later membranous bones, became intensely positive. In their centers deposits of calcium salts, at first granular but later coalescing to solid strands, made their appearance. Not a single instance of deposition of calcium salts in phosphatase-negative areas could be observed in more than 50 embryos

of various species and ages. Phosphatase-positive cartilage or connective tissue without any signs of calcification was seen quite often, but since no serial sections were made it is impossible to tell whether these positive areas did or did not contain centers of calcification at some other level.

In the bone tissue the osteoblasts remained positive for some time but soon lost their positivity in the more central, older portions of the bone, whereas in all areas where apposition was active, *i.e.*, under the periosteum and in the epiphyses, the osteoblasts were always strongly positive.

Teeth were examined only in a few rat embryos. Intense phosphatase reaction was obtained in the stratum intermedium of the enamel organ and also in the connective tissue of the pulp, especially in a layer immediately subjacent to the odontoblasts in regions where calcified dentin was present. The ameloblasts and the odontoblasts were negative or, in some cases, showed traces of positive staining.

It seems to be appropriate to include here the observations on one case of experimental rickets in a rat, although rickets is not a normal condition. The proximal tibial epiphysis was examined. Phosphatase was found to be present only in the hypertrophied zone of cartilage, while the remainder of the epiphyseal plate was negative. No calcification was present in the cartilage matrix. The osteoid borders of the bone trabeculae were negative for phosphatase but the osteoblasts lining them were positive. These observations are in full agreement with those of Freeman and McLean.

Bone formation under the influence of transplants of bladder epithelium was studied in 8 dogs, 4 guinea-pigs and 4 rabbits. Six pieces of the epithelium of the urinary bladder were transplanted in each animal to both rectus sheaths (three on each side) according to the technic of Huggins. They were spread as flatly as possible, the surface of the epithelium facing the skin. In addition, in 4 dogs small pieces of bladder epithelium were buried in the parenchyma of the liver and spleen and between the muscular layers of the stomach. The transplants were removed at regular intervals over a period of 10 weeks.

Epithelial cyst formation was observed in from 6 to 10 days at the sites of all transplants in dogs and in guinea-pigs and in 1 rabbit. In 3 rabbits the transplants disappeared.

Up to the point of completed cyst formation there was no difference between the histological pictures of the fascial and the intraparenchymatous transplants. In all transplants the epithelium retained its originally phosphatase-positive staining in the transplanted as well as in the regenerated portions. However, after the stage of cyst formation

the events took an entirely different course in transplants to the liver, the spleen and the stomach, on the one hand, and in transplants to the fascia, on the other hand.

In the liver, the spleen and in the wall of the stomach the cysts grew to a certain size, became rather distended, the epithelium became flattened and the entire structure was surrounded by a thick capsule of connective tissue. The epithelial lining remained positive throughout. No phosphatase was seen in the capsule.

In the fascial transplants on the 6th day the connective tissue subjacent to the newly formed epithelium showed a few scattered, strongly phosphatase-positive fibroblasts. In the next few days the number of these phosphatase-positive cells rapidly increased, and at the same time the fibrillar ground substance between them also became diffusely positive. By the 9th and 10th day a thick plaque of coarsely fibrillar, osteoidlike, intensely phosphatase-positive connective tissue was found under the newly formed epithelium. The thickness of the plaque was from 20 to 200 μ. It was not in immediate contact with the epithelium but was separated from it by an intermediate phosphatase-negative layer of connective tissue, about 20 μ in thickness. No reaction of the type described was seen under the old surviving epithelium of the transplant. By the 13th to the 20th day some of the densest strands in the centers of phosphatase-positive areas showed a granular deposit of calcium salts. The calcified areas rapidly extended and ramified. By the end of the 1st month well developed bone with hemopoietic marrow was seen in all transplants. The bone was surrounded by a wide zone of a rather cellular, intensely phosphatase-positive connective tissue, simulating the picture seen in embryonic osteogenesis. The entire process was essentially the same in dogs, guinea-pigs and in the rabbit.

As mentioned, bone formed only in the connective tissue underlying the newly formed epithelium. This is in complete agreement with the observations of Huggins.

The pathological material will be divided in three groups: (1) bone tumors; (2) tuberculosis; (3) calcification in hyaline connective tissue.

1. Bone Tumors

Four cases of bone-forming osteogenic sarcomas were observed. The pictures they presented were closely similar. All of the tumors were very strongly positive for phosphatase, especially in their most cellular, peripheral portions. Both the cells themselves and the fibrillar intercellular substance partook in the reaction. Granular deposits of

calcium salts were seen in strands of connective tissue within the positive areas. These deposits gradually coalesced until the entire stroma was outlined in black, with clear cells encased in the meshes. Two of the tumors had a marked tendency to sclerosis. In the sclerosing areas the reaction was far less strongly positive than in the cellular ones, except for groups of strongly positive cells growing within the vessels. Highly sclerotic, almost acellular areas were found to be entirely negative.

In addition to the cases mentioned, one case of osteoplastic metastasis of a cancer of the breast was observed. The tumor cells themselves were entirely negative for phosphatase, whereas the stroma was strongly positive, with extensive calcification and bone formation in the centers of positive areas.

Two giant cell tumors and two fibrosarcomas of bone, without new bone formation, were entirely negative for phosphatase, except for a few phosphatase-positive capillaries.

2. *Tuberculosis*

The material consisted of 42 rabbits, 58 guinea-pigs and 3 human cases. The rabbits were inoculated with 0.2 to 2 mg. of the culture of a bovine strain subcutaneously or intratracheally, the guinea-pigs with 2 mg. of a human strain subcutaneously. The animals were killed at regular intervals or allowed to die over a period of 55 weeks.

Observations on the three species will be given separately.

Rabbit. In the normal rabbit's lung phosphatase occurs in variable amounts in three different sites: first, the lining of the alveoli; second, the endothelium of blood vessels; third, in leukocytes and lymphocytes. The rabbit's neutrophils ("specials") are by no means as uniformly positive for phosphatase as those of the guinea-pig. Only a certain percentage of them are positive, and the ratio of positive to negative is usually much lower within the blood vessels than it is within the tissues. There is no morphological difference between the positive and negative cells; in fact, the two kinds are entirely indistinguishable from each other without the use of the phosphatase reaction. For the time being it is impossible to tell what factors control the number of positive and negative leukocytes. The above statements apply also to the lymphocytes. Within the blood stream most of them are negative, whereas in lymphocytic infiltrates the positive type prevails.

Early tuberculous granulation tissue without signs of necrosis was found to be entirely negative for phosphatase except for that contained in leukocytes and lymphocytes. As soon as necrosis set in phosphatase

appeared in the centers of the necrotic areas, first in the shape of an irregular, coarse network, later coalescing to a uniform, more or less rounded area of very intense phosphatase reaction. The enzyme was not carried in leukocytes since it appeared in all necrotic tubercles, regardless of whether they did or did not contain leukocytes. The positivity of the areas of necrosis did not last long as phosphatase soon disappeared from the centers, only to appear in the area of fresh necrosis in the peripheral zone of spread. In this way ever receding and enlarging rings of phosphatase-positive staining occupied the inner, necrotic area of the zone of spread. If the process came to a standstill, phosphatase disappeared altogether.

The intensity of phosphatase-positive staining in the early necroses was so high that it is impossible to account for the amount of enzyme present in them from purely local sources. As mentioned, phosphatase is not carried in by leukocytes. The synthesis of such a highly specific enzyme by a necrotic mass is improbable. The most probable explanation seems to be the adsorption of the enzyme from the blood and from the tissue fluids by the fresh necrotic material, owing to some physicochemical property of the latter at a certain stage of development. At a later stage this property is lost and consequently the enzyme is eluted from the lesion. It should be mentioned here that in two rabbits necrosis of the lung and liver were produced by the intraparenchymatous injection of alcohol. No phosphatase was found in areas of necrosis produced by this method.

The changes mentioned were remarkably uniform in the pulmonary lesions of all animals except two. In the latter cases phosphatase failed to appear in the necrotic areas. The phosphatase picture of lesions in other organs was far less regular. Most areas of necrosis were found to be free of phosphatase, regardless of their age; and even when phosphatase was present, the reaction was much fainter than in the lesions of the lungs.

Calcification was observed in 15 cases out of 42. It involved pulmonary lesions only, although extensive tuberculosis of the spleen, liver and kidneys was present in many of the cases. The earliest date at which calcification could be demonstrated was 38 days after inoculation (average: 101 days).

If calcification took place, its earliest traces invariably occurred in the center of some phosphatase-positive area. Calcification did not alter the peripheral shift of phosphatase as described. Phosphatase receded in the shape of a ring from the central calcification in the typical way. In the peripheral positive area new centers of calcification often developed which, if they coalesced, led to the formation of im-

perfect concentric calcareous shells. This pattern of concentric rings is well known from x-ray studies on human tuberculosis.

Guinea-Pig. The normal lung of the guinea-pig does not contain any phosphatase at all, except in the scattered leukocytes which are constantly and strongly positive in this species and in the epithelial lining of some of the bronchi.

Tuberculous granulation tissue as well as areas of necrosis were entirely free of phosphatase, except for the leukocytes. In my series of guinea-pigs the lesions were of a markedly exudative character and the tubercles in practically all cases were densely infiltrated by leukocytes. The latter often formed strongly phosphatase-positive abscesses in the centers of the lesions. After the breakdown of the leukocytes the enzyme diffused in all directions. All the phosphatase in guinea-pig tubercles comes from leukocytes. These data are in complete agreement with those presented by Takeuchi and Takamatsu.

Calcification was observed in only 3 cases out of 58. It involved both pulmonary and splenic lesions. The earliest date at which calcification could be found was 55 days after inoculation. It always occurred in the centers of necrotic areas. Phosphatase could be seen around some of the calcified centers, but owing to the small number of cases no further conclusions can be drawn.

Man. The normal human lung contains extremely variable amounts of phosphatase in three sites: first, in the lining of the alveoli; second, in the endothelial lining of capillaries; third, in the epithelium of the bronchi. Human leukocytes are free from phosphatase.

The number of human cases observed being only three, no definite conclusions can be drawn as to the phosphatase picture in human tuberculosis. Tuberculous granulation tissue was found to contain no phosphatase. There was a marked perifocal intensification of the reaction in the alveolar lining. In one case the outer fibrous wall of a cavity was found to be positive. Takeuchi and Takamatsu made practically the same observations. Calcification was observed in one old, encapsulated lesion, with no trace of phosphatase around it.

3. *Calcification of Hyaline Connective Tissue*

This group includes 6 examples of early arteriosclerosis of the aorta; 2 of sclerosing and calcifying pyelonephritis in human beings and 1 in a rabbit; 2 calcifying goiters and 1 calcifying islet cell tumor of the pancreas. In all of these, granular calcium salt deposits were observed in very dense, almost acellular, connective tissue. No trace of phosphatase was found around any of the granules of calcification.

As mentioned, sections of practically all specimens were also stained

by my technic for the demonstration of acid phosphatase. In no instance could the presence of this enzyme in or around calcareous deposits be demonstrated.

Comment

Calcification of living or recently necrosed tissue seems to start invariably in the centers of intensely phosphatase-positive areas. On the other hand, calcification of sclerosed, hyaline connective tissue apparently can and does occur without phosphatase action. Although it is possible that the latter type of calcification may be initiated by phosphatase action followed by a rapid disappearance of the enzyme, the fact that phosphatase was not found in a single instance around deposits of calcium in hyaline connective tissue is against such an assumption. Obviously there are other mechanisms besides phosphatase action capable of raising the $[Ca^{++}] \times [PO_4^{\equiv}]$ product beyond the critical level. The results of Ross clearly show that under certain conditions tissues may calcify far below the $[Ca^{++}] \times [PO_4^{\equiv}]$ value of 3.3×10^{-6}, required for the calcification of bone. The nature of the mechanism involved in calcification at low $[Ca^{++}] \times [PO_4^{\equiv}]$ products is not understood at present. One possibility, unexamined so far, would be the presence of some protein with a higher dissociation constant than that of plasma proteins, resulting in a higher local concentration of calcium ions at the same level of total calcium.

An interesting problem is the source of phosphatase in the connective tissue around transplants of bladder epithelium. The two possibilities considered are: (1) simple diffusion of the enzyme (and, according to Huggins, of calcium and phosphate) through the thin layer of newly formed epithelium into the surrounding tissue, and (2) specific inductive power of the bladder epithelium on certain kinds of connective tissue, resulting in the proliferation of phosphatase-producing fibrocytes. The theory of simple diffusion does not explain the fact that calcification and bone formation is regularly produced at certain sites (fascia), while consistently negative results are obtained at other sites (liver, spleen, stomach), unless the presence of an antiphosphatase in these latter organs is hypothecated. On the other hand, the theory of specific induction would fit well into proved facts of embryology. Gruenwald published striking instances of strictly region-bound inducibility of the mesenchyme by the growing wolffian duct.

An attempt was made to solve this problem by transplanting other, normally phosphatase-positive, kinds of epithelium to the fascia and by observing whether or not phosphatase and bone formation would

appear around them. In 3 dogs pieces of duodenal mucosa and in 5 more dogs and in 3 guinea-pigs pieces of the epididymis (finely minced to provide for a large surface of contact) were transplanted to the rectus sheath. The transplants of duodenal mucosa were complete failures. They all disappeared within 3 weeks. The transplants of epididymis took well and grew considerably in 10 weeks. Many epithelium-lined cysts were observed. Although the epithelium in places retained fully its phosphatase-positive character, no phatase and no bone formation were observed in any of the plants. The results of these experiments seem to support the t of specific induction rather than that of diffusion. Induction of bo... formation by bladder epithelium seems to be a favorable case in which it is possible to trace the induction mechanism one step farther back than in most other instances.

Summary

A new microtechnical method for the simultaneous demonstration of preformed calcium salt deposits and of sites of phosphatase activity is presented, together with observations made with this method on normal and pathological calcification.

Calcification of living or recently necrosed tissues seems invariably to involve phosphatase activity. On the other hand, calcification of hyaline connective tissue occurs without any phosphatase action. An attempt is made to explain the difference between the mechanisms of these two types of calcification.

Acid phosphatase plays no rôle in calcification.

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[*Illustrations follow*]

DESCRIPTION OF PLATE

PLATE 24

Calcifications, black; sites of phosphatase activity, purplish red; nuclei and ground substance of cartilage, green-blue.

FIG. 1. Parasagittal section of a rat embryo, 18 mm. long, showing processes of vertebrae. Positive reaction in the perichondrium. × 36.

FIG. 2. Lower incisor of a newborn rat. × 30.

FIG. 3. Bone-producing transplant of bladder mucosa. Positive reaction in the basal layer of the epithelium and in a thick strand of connective tissue in the upper right part of the field. The bone is surrounded by a wide zone of positive reaction. × 36.

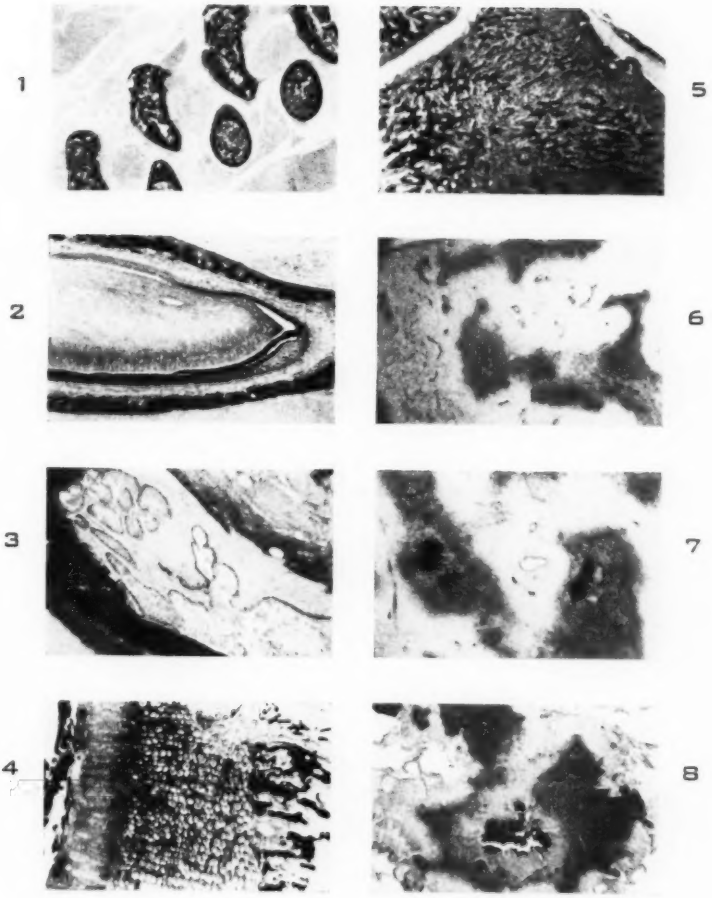
FIG. 4. Upper tibial epiphysis of a rachitic rat. The zone of hypertrophied cartilage is strongly positive for phosphatase. × 48.

FIG. 5. Osteogenic sarcoma of the tibia. The tumor is strongly positive, the surrounding connective tissue is negative. × 36.

FIG. 6. Phosphatase reaction in the center of an early necrotic tubercle in the rabbit's lung. × 36.

FIG. 7. Calcification in centers of phosphatase-positive areas (rabbit's lung). × 36.

FIG. 8. Peripheral recession of phosphatase reaction with secondary calcification at the periphery (rabbit's lung). × 30.



Gomori

Calcification and Phosphatase

THE NATURE OF THE RENAL LESION WITH THE SULFONAMIDES AND ITS PREVENTION WITH UREA *

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A review of the reported renal changes exhibited by the various sulfonamide compounds in animals and man reveals, with minor differences, a constant pathologic picture.[1-10] The cause of these changes has not been clearly demonstrated and the relative rôles of primary chemical injury and of trauma secondary to mechanical precipitation have not been adequately evaluated.[11-13]

In a study of the *in vitro* solubility of acetylsulfathiazole and acetylsulfapyridine in urine, Curtis and Sobin [14] found that at a given pH solubility increased in proportion to the specific gravity of the urine. From both theoretical considerations and experimental data it was pointed out that the factor responsible for the increase in solubility of these materials in more concentrated urines was probably an increasing amount of urea. It was at once apparent that what was true in the test tube might also be true in the animal body. The *in vivo* use of urea might, therefore, enable a distinction to be drawn between the primary nephrotoxic properties of these drugs and mechanical irritative effects resulting from their intrarenal precipitation.

METHOD

Analysis of precipitated material found in the renal tract when the sulfonamide drugs are administered over an extended period to man and animals has shown a high percentage of acetylated derivatives.[15-19] Oral administration of these acetylated drugs is unsatisfactory because of insolubility and erratic absorption. Accordingly, sodium acetylsulfapyridine was prepared by the method of Marshall, Bratton and Litchfield [20] for the sodium sulfonamides.†

Forty-eight adult white male rats were divided into four groups of 12 and treated as follows: groups 1 and 2 were each given 1 mg. of sodium acetylsulfapyridine per gram of body weight, but in addition group 2 received 5 mg. of urea per gram of body weight. Groups 3 and 4 received 3 mg. of sodium acetylsulfapyridine per gram of body weight and group 4 received in addition 10 mg. of urea per gram of body weight. Untreated rats of the same age were used as controls.

* Supported in part by a grant from the Committee on Scientific Research of the American Medical Association.

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† Attempts to prepare crystalline sodium acetylsulfathiazole by this method were unsuccessful and sodium acetylsulfapyridine alone was used in these experiments.

The drugs were administered in solution through a stomach tube. The volume of solution of sodium acetylsulfapyridine was calculated on the basis of rat weight and the urea was added. Animals were treated over periods of 7 to 14 days; some were treated daily and others intermittently. Throughout the period of observation the daily water intake and the weight were noted.

Tissue sections were prepared in the usual manner with the precaution that previous to embedding in paraffin all solutions were saturated with acetylsulfapyridine. This included the formaldehyde fixative and the various dehydrating and clearing agents. Such a procedure eliminated the necessity of a rapid and special method for the demonstration of intrarenal sulfonamides [3, 21] (Fig. 1). Serial sections were made of both kidneys, and a systematic examination was carried out.

Observations

Throughout the progress of the study it was noted that animals treated with sodium acetylsulfapyridine alone, frequently presented a

Table I

Fluid Intake of a 10-Day Experiment
(computed as cc. per gm. of rat per day)

	Day										
	0	1	2	3	4	5	6	7	8	9	10
Group A*	0.27	0.20	0.18	0.22	0.23	0.21	0.16	0.28	0.13	0.24	0.25
Group B†	0.25	0.31	0.25	0.17	0.20	0.18	0.14	0.29	0.19	0.18	0.37
Group C‡	0.24	0.36	0.17	0.20	0.19	0.17	0.12	0.24	0.19	?	0.12

* Sodium acetylsulfapyridine (3 mg. per gm. of rat per day) without urea. (Animals receiving 5 mg. of urea in addition to 1 mg. of sodium acetylsulfapyridine per gm. of rat showed no increased water intake over animals not receiving urea.)

† Sodium acetylsulfapyridine (3 mg. per gm. of rat per day) with urea (10 mg. per gm. of rat per day).

‡ Controls.

hematuria which was not constant from day to day. Hematuria was not encountered in any animal receiving urea along with sodium acetylsulfapyridine. The animals not receiving urea frequently appeared ill, listless and took their food poorly. This was not true of the urea group.

A study of the daily water intake (computed as cc. per gram of rat per day) showed no increase in the urea-treated animals (Table I). Inasmuch as the interval from the beginning to the end of the experiment was sufficient to allow for water balance, the urea effect cannot be attributed to diuresis.

The impression gained from the hematuria was confirmed upon

examination of the kidney sections. Two types of foreign material were found in many of the kidney sections of the animals treated with sodium acetylsulfapyridine alone: intrarenal, nonstaining crystals (Fig. 1) and bluish staining (with hematoxylin) intratubular masses (Fig. 2). In animals receiving 1 mg. per gram of body weight of the drug, calculi * were noted in slightly less than half of the kidney sections; in those receiving 3 mg. per gram of body weight of the drug, they were noted in about 90 per cent of the cases. Calculi were not found in the kidney sections of any animal in the urea-treated groups.

A low-power magnification of sagittal kidney sections showed most of the calculi to be at the corticomedullary junction; however, deposition in other areas of both cortex and medulla was observed. Precise localization of calculi was difficult. Under higher magnification many were found within Henle's loops, convoluted tubules and collecting ducts, but it was impossible to differentiate proximal from distal tubules in the sections studied. Antopol [4] and Lehr and Antopol [9] were able to recognize distal convoluted tubules as the site of calcification.

In order to determine the composition of the intratubular blue-staining masses, attempts were made to stain the acetylsulfonamide compounds. With hematoxylin, silver stains (Fig. 1) and fat stains it was impossible to stain acetylsulfapyridine. The basophilic nature of the calculi with hematoxylin further suggested that some substance other than acetylsulfapyridine was present. Use of von Kossa's stain for calcium indicated that this was true and that the calcium content was high (Fig. 3). Calcium deposition was seen to occur in at least two ways: as small calcific deposits (Fig. 4) and by lamellation upon these small foci (Fig. 2).

The mechanism of calculus formation is important. In the dosage used it took 9 or 10 days before calcium could be found in mass deposits. In sections taken from animals treated for 10 days and then sacrificed, liberal amounts of calcium were noted (Figs. 2 and 3). Animals sacrificed after 7 days of treatment showed only focal areas of severe tubular degeneration (Fig. 5). These tubular epithelial cells were markedly edematous with disappearance of former cell margins and the formation of a granular protoplasmic mass. The nuclei were swollen, and fragmented nuclear material was recognizable. These

* A distinction must be made between the various terms applied to foreign material found in the renal tract with sulfonamide therapy. *Concretion, calculus, urolith* and *stone* have been used indiscriminately. We reserve *calculus* for those bodies with a mineral content, especially calcium, and *concretion* for any solid body. *Urolith* and *stone* by derivation imply a mineral or calcium content. Precipitated sulfonamides will be referred to as such, or as concretions.

areas took a bluish cast with hematoxylin and eosin stains, possibly due to diffuse chromatin. Many such areas were found in tissues showing abundant concretions. In the latter tissues, routine stains showed tiny basophilic areas and corresponding silver stains showed foci of calcium deposition (Fig. 6). These areas of focal calcium deposition also showed iron deposition by the Prussian-blue reaction. In the tissues of animals treated only 7 days these areas of focal necrosis showed no calcium deposition. Areas of focal regressive change were not encountered in urea-treated animals or in normal controls.

Fat stains did not demonstrate degenerative fatty infiltration in either severely injured kidneys with focal necrosis and other regressive changes or in the kidneys of animals protected with urea.

Distinction must be made between areas of tubular necrosis with secondary calcification as described above and areas of tubular damage from pressure necrosis by calculi already present (Fig. 7). This latter type of calculus was formed by deposition of calcium salts upon necrotic epithelial cells free in the tubules. The epithelium of the tubule was seen to be intact except where the foreign body was in contact with it. In some sections a leukocytic response was noted at the areas of contact of calculus and epithelium. Although calcification upon an organic nidus can occur either *in situ* or free on necrotic epithelial cells, the significance of such calcification does not differ in either case.

Renal changes other than areas of focal regressive change and calculus formation were similar to those adequately described and illustrated by others and will not be considered in detail here. Cloudy swelling, focal and generalized interstitial cellular infiltration, pyelitis and dilatation of the whole nephron, including compression of the glomerular tuft and intracapsular deposition of foreign material, have been seen in varying degree only in the animals treated with acetylsulfapyridine. Thickening of the basement membrane of Bowman's capsule, with foreign material in the capsular space, has been noted in human tissues; [7,9] minor changes of this type have also been seen in this material.

Discussion

The prevention of the renal calculi resulting from administration of sodium acetylsulfapyridine by means of simultaneously administering urea depends upon a solvent effect of urea on both free [22] and acetylated sulfonamide compounds.[14] That such solvent effect occurs in both test tube and animal body is some evidence of its specificity.

Originally, the possibility of calcium deposition upon precipitated intratubular sulfonamide was suggested by Antopol and Robinson.[23]

Recently this view has been again expressed by Lederer and Rosenblatt [8] from a review of human material. That intrarenal calcium deposition did occur with administration of free and acetylated sulfonamide compounds was subsequently demonstrated by Antopol and co-workers.[3-5, 9, 11] They have termed this lesion *calcifying nephrosis*. Details of the pathogenesis and significance of such calcification have not been previously reported. With the dosage used in these experiments it takes about 10 days for calcification to occur. Antopol, Lehr, Churg and Sprinz [5] have reported calcification in 24 hours when massive intrarenal precipitation of sulfonamides occurs as a result of parenteral therapy with the soluble sodium salts.

In a study of the renal pathology resulting from mercuric chloride poisoning, Harmon [24] reported changes similar to those described above. Regressive changes ranging from severe parenchymatous degeneration to necrosis of the tubular epithelium were distributed from proximal convoluted tubules to collecting tubules. Glomerular changes were not found. Two types of cell death were present: (1) simple coagulation necrosis without edema, with pyknosis or disappearance of nuclei, attributed to the primary nephrotoxic properties of mercury; and (2) marked cellular swelling with large vesicular fragmented nuclei, possibly associated with acid intoxication and the resulting anuria. Calcification of the necrotic epithelium occurred in free masses in the tubular lumen and in the tubular lining cells *in situ*. No calcification of apparently living cells was found. Photographs showing calcium deposition as a result of mercurial intoxication are indistinguishable from those seen with sodium acetylsulfapyridine.

Smetana [25] has designated the renal pathology resulting from carbon tetrachloride intoxication as *nephrosis* characterized by "distention of the spaces of Bowman with albuminous precipitate, with swelling of the lining cells, swelling and vacuolation of the cells of the proximal convoluted tubules, degeneration and necrosis of the cells of the distal convoluted tubules and those of the loops of Henle, with desquamation, and by the presence of granular, hyaline and cellular casts in the tubules, with plugging of their lumina. Concretions are present whose nature and significance are obscure." These concretions were either yellow-green or intense blue with hematoxylin and eosin stains, and stained black with von Kossa's stain. Although Smetana believed that calcification of necrotic tubular cells, as commonly observed in bichloride of mercury poisoning, is not a feature of carbon tetrachloride poisoning, the morphology and staining qualities of the concretions which he described leave no doubt that deposition of calcium had occurred.

Animal experimentation indicates that the renal injury in experimental bismuth intoxication is not dissimilar from that described for mercurial poisoning.[26] Abundant calcium in the kidneys of rabbits treated with bismuth has been recently reported by Kroll, Arens, Mesirow, Strauss and Necheles.[27]

In the absence of intrarenal precipitation of free and acetylsulfonamide compounds, significant alterations of renal architecture are not found. This suggests that a direct relationship exists between calculus formation and precipitated sulfonamides, even though crystalline sulfonamide is not recognizable in these calcific masses. Our interest in the renal changes with the heavy metals and carbon tetrachloride is in the fact that chemical injury, with associated regressive changes leading to epithelial tissue death, may be followed by deposition of calcium upon the nonviable tissues as a focus. We believe that the pathogenesis of renal calcification observed with sulfonamide drugs is by a similar mechanism and is a general response of the kidney to injury. Mechanical irritation occurs as a result of precipitation of these materials; the resulting tissue injury may be severe enough to produce local necrosis. It is upon these areas of necrotic tissue that calcium deposition occurs.

Reference has already been made to reports on the renal changes aside from calculus formation.[1-10] The distended tubular system has been recognized as hydronephrotic; the type and degree of such a process should depend on the amount of precipitated sulfonamide (free and acetylated) blocking the tubular system. Comparable lesions have been produced by ligation of the ureteropelvic junction.[28] Interstitial inflammatory changes and thickening of the parietal layer of Bowman's membrane have also been observed with this same procedure. As yet it has not been demonstrated that these changes noted with sulfonamide therapy are independent of tubular block and hydronephrosis *per se*. Focal and diffuse parenchymatous degeneration is adequately explained by mechanical trauma, and chemical trauma has not been established.

Of additional importance are the renal changes that result from administration of amounts of sulfonamide drug below that which produce intrarenal crystal formation by microscopic examination.[11-13] These changes are similar to those described above. Prior to the utilization of urea in preventing precipitation of sulfonamide in the renal tract, this method of minimal dosage was the only one available to determine chemical nephrotoxic properties of these drugs. There is no positive evidence that crystal formation has not occurred with even the relatively small amounts of these drugs necessary to produce renal

lesions. It is certain that simultaneous administration of urea does prevent the pathologic changes in the kidney exhibited by these drugs.

The extremely rare renal lesions with sulfanilamide and neoprontosil have been associated with demonstrable crystalline masses in the kidney.[29] The recent report of Lehr and Antopol [9] on renal complications with sulfadiazine stated that "it is the physical factor of poor solubility, as generally created by acetylation, rather than the chemical change of the compound which accounts for the kidney damage." We subscribe to this view.

Focal necrosis has been seen in human material that presented the clinical syndrome of sulfathiazole toxicity. No counterpart of this symptom-complex has as yet been encountered in animals and it is doubtful that the mechanism of production of lesions is similar.

A consideration of the differential function of the various parts of the nephron, as outlined by Edwards,[30] may explain the reported differences in the site of precipitation of the different sulfonamides.[16] It may further explain the susceptibility to renal complications from the use of sulfonamides in patients with previous renal injury.

Conclusions

1. Intrarenal foreign material following sulfonamide drug therapy is of two types: (1) precipitated sulfonamide and its acetylated products; (2) cellular débris, with calcium and iron deposition around or on this material.

2. Urea simultaneously administered with sodium acetylsulfapyridine will prevent the precipitation of sulfonamides and the formation of renal calculi in rats.

3. The action of urea is independent of a diuretic effect and depends upon a specific solvent effect on acetylsulfapyridine.

4. The nephrotoxic properties of acetylsulfapyridine are mechanical in nature, and result from precipitation of the drug in the renal tract.

5. Calcification in the kidney and the resultant calculus formation in sulfonamide-treated animals is dependent upon local tissue damage and the secondary deposition of calcium and iron upon focal, nonviable structures.

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[*Illustrations follow*]

DESCRIPTION OF PLATES

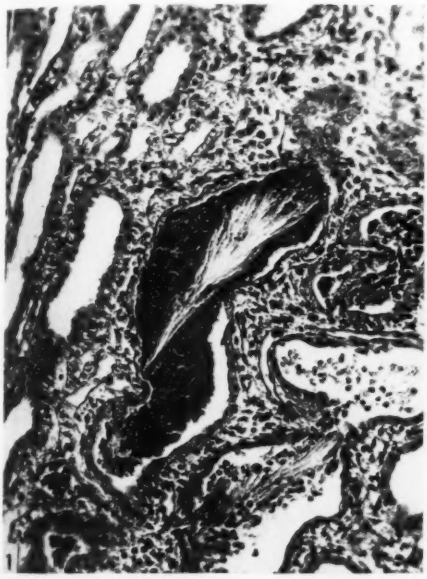
PLATE 25

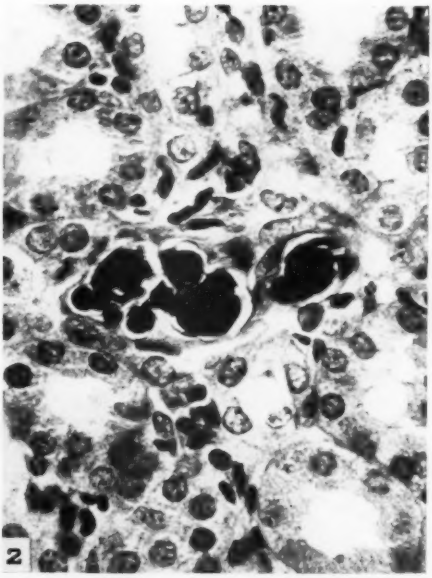
FIG. 1. Cortex of kidney of rat showing sheaths of nonstaining acetylsulfapyridine crystals in collecting ducts. Packed erythrocytes surround the crystals in the central tubule; leukocytes can be seen at the lower border of the packed cells. Intratubular leukocytes can be more clearly seen in the sheath at the lower right, as well as an early interstitial leukocytic response at the contact area of the sheath and the injured duct wall. Masson and von Kossa's stains. × 145.

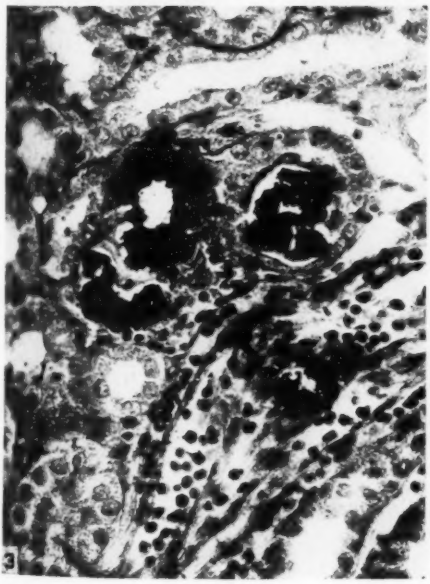
FIG. 2. Intratubular blue-staining masses in cortex of kidney of rat. There is complete absence of tubular epithelium. Calculi show concentric deposition of calcific material. The tubules surrounding the concretions are intact. Hematoxylin and eosin stain. × 575.

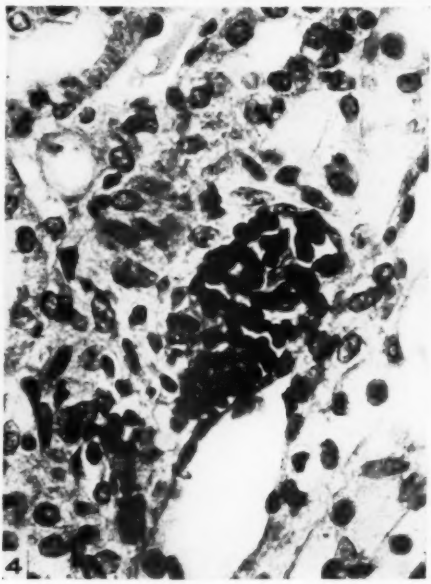
FIG. 3. Calcium deposition in intratubular concretions. Preparation as in Figure 1. × 325.

FIG. 4. Corticomedullary junction of kidney of rat with multiple small foci of calcium deposition in an area of tubular necrosis. Preparation as in Figure 2. × 510.









Sobin, Aronberg and Rolnick

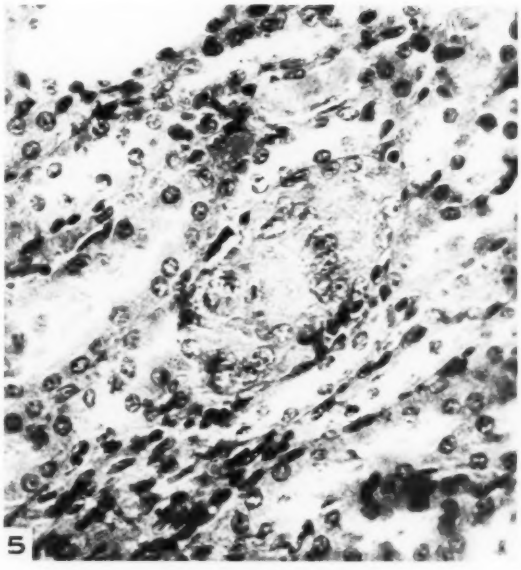
Renal Lesions with the Sulfonamides

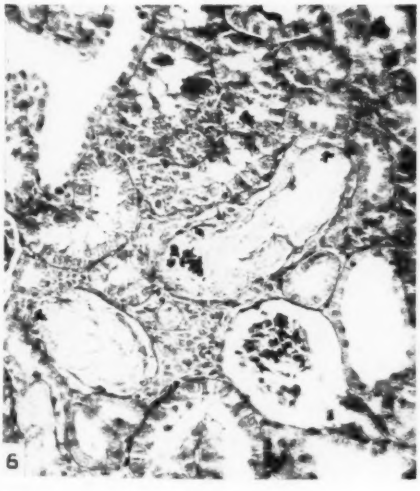
PLATE 26

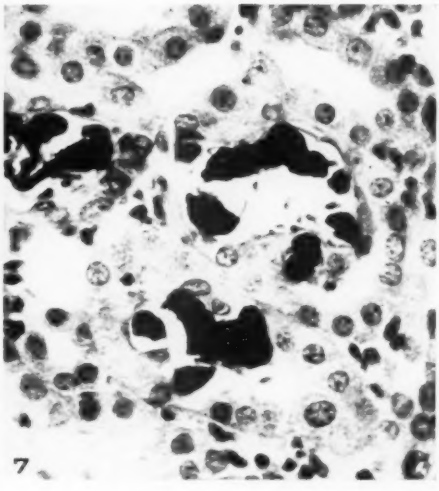
FIG. 5. Focal necrosis of the tubular epithelium. This area takes a bluish stain with hematoxylin and eosin. Swelling of nuclei, loss of cell boundaries and granularity of cytoplasm are seen. Hematoxylin and eosin stain. × 400.

FIG. 6. Early calcium deposition in an area of focal necrosis. There is a severe degenerative change of the tubular epithelium with scattered early foci of calcium deposition. Masson and von Kossa's stains. × 210.

FIG. 7. Tubular injury secondary to concretion formation. There is a loss of continuity of tubular epithelium at the area of contact of preformed intratubular concretions with the tubular wall. The epithelium is otherwise intact. Hematoxylin and eosin stain. × 435.







Sobin, Aronberg and Rolnick Renal Lesions with the Sulfonamides

ERYTHROPHAGOCYTOSIS AND HEMOSIDEROSIS IN THE LIVER AND SPLEEN IN SICKLE CELL DISEASE *

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Abnormal destruction of erythrocytes by the cells of the reticulo-endothelial system is characteristic of many hemolytic anemias. However, these cells seldom exhibit morphologic evidence of erythrophagocytosis. It has been seen occasionally in pernicious anemia, hemolytic jaundice, sickle cell anemia, icterus gravis neonatorum and Weil's disease. The degree to which this erythrophagocytosis is apparent differs in various parts of the reticulo-endothelial system. The spleen, as a rule, shows very little evidence of this process.[1]

The pathologic changes of the different organs in sickle cell anemia have been repeatedly summarized.[2-6] It has been stated that the Kupffer cells in the liver frequently exhibit phagocytized red blood cells.[2,5,7] The changes in the spleen also have been thoroughly investigated.[8] The details, however, as to the frequency and extent of these processes are not yet established.

The case of sickle cell disease herein reported is illustrative of a disproportional degree of erythrophagocytosis in the liver and of hemosiderosis in the spleen. A survey of the occurrence of these processes in a number of cases examined at necropsy, with an attempt to correlate the splenic and hepatic changes, is also included. A similar analysis of the reported cases in the literature with complete necropsy data has also been attempted.

MATERIAL

From 4,094 postmortem examinations at Charity Hospital of Louisiana from January 1, 1939, to January 1, 1942, 12 cases of sickle cell disease were available for study. Cases with incomplete data were excluded. Data pertaining to the age, sex, size of the spleen and liver, and the degree of erythrophagocytosis and hemosiderosis were tabulated. In addition to the routine hematoxylin and eosin staining, the presence of hemosiderin was tested by the Turnbull-blue reaction.[9]

Since the anemia was not established in all cases, the term "sickle cell disease" will be used, rather than "sickle cell anemia."

REPORT OF ILLUSTRATIVE CASE

A colored male, 14 years of age (see case no. 3, Table II), was admitted with cough, and edema of the face. He gave a history of having had heart trouble for 1 year. Three years prior to admission he had had rheumatic fever.

* Received for publication, June 1, 1942.

Examination revealed a poorly nourished, but fairly well developed patient with a temperature of 99.6° F.; pulse, 110; respiration, 26; blood pressure, 116/40. The heart was markedly enlarged. A blowing systolic apical murmur was heard. The liver was markedly enlarged. On the anterior aspect of the legs there were numerous small scars present.

Laboratory Findings. The blood examination was as follows: Hemoglobin, 5.8 gm. per 100 cc., 40% (Hellige); red blood cells, 1,780,000; white blood cells, 19,000; nucleated red cells, 60,000, and platelets, 500,000 per cmm.; hematocrit, 21.5 (Wintrobe); polymorphonuclears, 48 per cent; eosinophils, 4 per cent; monocytes, 9 per cent; lymphocytes, 25 per cent; promyelocytes, 2 per cent; myelocytes, 9 per cent, and metamyelocytes, 3 per cent. The wet preparation showed 90 per cent of the red blood cells to be sickle-shaped. The fragility of the red cells was normal. A sternal puncture revealed a hyperplastic, pronormoblastic marrow. Clotting time and coagulation time (Lee-White) were within normal limits. The urine showed no deviation from the normal. The icterus index was 66; the van den Bergh test showed 7.6 mg. of bilirubin per 100 cc. (direct method). Four days later the van den Bergh test gave 25 mg. of bilirubin per 100 cc. (direct method). The Kline flocculation test was negative. On several occasions the sputum was negative for acid-fast bacilli. Roentgenograms of the chest showed far advanced bilateral pulmonary tuberculosis and cardiac enlargement. The electrocardiograph showed evidence of myocardial disease typical of rheumatic mitral stenosis.

Clinical Course. Patient became progressively more anemic and developed jaundice, dyspnea, and pitting edema of both legs. His temperature became of septic type, and he expired 77 days after admission to the hospital.

Necropsy Findings

There was marked edema of the face and lower extremities. Both legs presented numerous scars on the anterior aspects. The heart weighed 500 gm. and showed marked right ventricular hypertrophy and mitral stenosis. The lungs presented a far advanced exudative tuberculosis with numerous cavities. Enlarged caseous hilar nodes were present. The spleen was small, firm, purplish in color and weighed 24 gm. Its cut surfaces were fibrous. The liver weighed 3600 gm., and the cut surfaces revealed diffusely scattered grayish yellow areas measuring 1 mm. in diameter. The kidneys were markedly enlarged, weighing together 720 gm. The sternum, thoracic vertebrae and the middle portion of the femur presented red, active marrow.

Histologic Examination

A large proportion of the red blood cells were sickle-shaped in all organs. The sinusoids of the liver were markedly distended; the Kupffer cells were enlarged and their cytoplasm contained numerous sickle-shaped red blood cells (Fig. 4). The liver cells were compressed and contained finely granular cytoplasm, with well stained nuclei. Occasional small areas of extramedullary myelopoiesis were seen. Some bile capillaries contained bile plugs. The spleen showed a marked increase in connective tissue. Around the blood vessels large amounts of dark brownish green pigment were deposited, which varied in intensity. Also aggregations of dark blue incrustations were evident. The

lymph follicles were small and without germinal centers. Some sinusoids were distended with sickle-shaped red blood cells. The walls of the sinusoids were thickened and the sinus endothelial cells were conspicuous, occasionally containing phagocytized red blood cells. In addition to the tuberculous lesions, the lungs showed large alveolar phagocytes containing sickle-shaped red blood cells. The kidneys showed hyperemia, interstitial edema and small areas of erythropoiesis. Active pronormoblastic erythropoiesis was seen in the bone marrow. The mesenteric lymph nodes presented small follicles and markedly distended sinusoids, with active erythrophagocytosis by sinusoidal cells. The Turnbull-blue stain was strongly positive in the spleen and slightly positive in the liver, kidneys, bone marrow and mesenteric lymph nodes.

Summary of Case

In the case presented the spleen was small and fibrous with calcium and iron incrustations. The liver was greatly enlarged (Fig. 6) and exhibited a marked degree of erythrophagocytosis. The various portions of the reticulo-endothelial system appeared to be in quite different functional stages. The splenic reticulo-endothelium appeared to be inactive while the Kupffer cells exhibited marked hyperactivity.

Observations, Including Additional Cases

The 12 cases were divided into two groups according to the size of the spleen. The first group (Table I) included 7 cases in which the spleen was of normal weight or enlarged (average 286.4 gm.). The

Table I

Cases of Sickle Cell Disease with Spleens of Normal Weight, or Larger

Case no.	Age	Sex	Spleen gm.	Liver gm.	Liver-spleen ratio	Erythrophagocytosis		Hemosiderosis		Complicating disease
						Spleen	Liver	Spleen	Liver	
1. W. S.	28	M	140	1535	10.96	—	+	—	—	Brain abscess
2. E. H.	47	M	160	1720	10.75	—	—	+	+	Acute thyrotoxicosis
3. L. M.	40	F	225	2400	9.60	+	—	+	—	Typhoid fever
4. M. S.	6	M	70	640	9.14	—	+	+	+	Wilms' tumor
5. A. L.	49	M	150	2200	14.66	—	++	++	—	Type 23 pneumococcus meningitis
6. J. W.	54	M	190	1790	9.42	+	++	—	—	Hypertension
7. Z. K.	17	M	1070	1650	1.54	+	++	+	—	Abdominal crisis
Average	34.4		286.4	1705						

livers of this group were of normal weight or moderately enlarged (average 1705 gm.). The average age of the patients in this group was 34.4 years, the ages ranging from 6 to 54 years.

The second group (Table II) consisted of 5 cases in which the spleen was small (average 19.7 gm.). The livers varied from a normal size to markedly enlarged (average 2106 gm.). The average age was 19 years, the ages ranging from 14 to 39 years.

Because of the wide age range in both groups the hepatic-splenic ratio was computed according to the method given by Ahronheim, [10]

TABLE II

Cases of Sickle Cell Disease with Small Spleens

Case no.	Age	Sex	Spleen gm.	Liver gm.	Liver-spleen ratio	Erythrophagocytosis		Hemosiderosis		Complicating disease
						Spleen	Liver	Spleen	Liver	
1. E. L.	22	M	5.4	1950	361.1	—	+++	—	+	Abdominal crisis
2. E. R.	39	F	7.0	1250	178.6	—	—	+++	—	
3. C. O. H.	14	M	24.0	3600	150.0	++	++	+++	+	Tuberculosis
4. W. M. L.	18	F	50.0	3080	61.6	—	+	+++	—	Postpartum thrombophlebitis
5. F. W.	2	F	12.0	650	54.2	+	—	++	—	Rheumatic fever
Average	19.0		19.68	2106						

who found that the normal ratio between hepatic and splenic weight is 9.3:1. The ratios for the cases tabulated in Table I were close to or slightly above this value, with two exceptions—cases nos. 5 and 7. Case no. 4, a boy of 6 years, showed absolute values above the normal for his age, [11] but the hepatic-splenic ratio was within normal limits. The cases tabulated in the second group showed very high values for the ratio, indicating a marked diminution of the spleen and enlargement of the liver in all instances.

Histopathology of the Liver

The livers were enlarged, reddish brown and firm. Histologic study revealed marked congestion, the sinusoids being distended and filled with sickle-shaped red blood cells. The Kupffer cells were swollen and exhibited varying degrees of erythrophagocytosis. This was most marked in case no. 1, Table II (Fig. 4). The liver cords were regularly arranged and the parenchymal cells were compressed or slightly swollen and granular. Infrequently lipoidosis in varying degrees was seen.

Histopathology of the Enlarged and Normal-Sized Spleens

The spleens of the first group revealed a marked congestion of the sinusoids and venous sinuses, which were filled with sickle-shaped red blood cells. The lining cells of the sinusoids formed thin, irregular, netlike structures. The central arteries were also markedly dilated and the follicles were without germinal centers. Often there were pools of extravasated blood around the follicles. This was most marked in case no. 7, Table I, in which the spleen weighed 1070 gm. (Fig 5). Varying amounts of iron-containing pigment were found. The reticulum showed in places a somewhat thicker fibrillar network, upon which hemosiderin was deposited in the form of myceliumlike threads (Fig. 1). These changes were often confined to the vicinity of the small blood vessels. Around the periadventitial tissue the reticulum coalesced to form a thick hyaline network, and here the Turnbull-blue stain revealed thick rods of deposited iron (Fig. 2). In irregular areas calcium deposits were also present and small hemorrhages and old infarcts of small size were often seen.

Histopathology of the Fibrotic Spleens

There was an increase of hyaline connective tissue and widening of the trabeculae in the fibrotic spleens. In this connective tissue, irregular areas of dark bluish green or brownish clumps, often arranged in parallel rods resembling bamboo sticks, were formed. In other instances, the deposits formed structureless bluish brown masses (Fig. 3). The walls of the sinusoids were thickened, and their endothelial lining cells were flattened. Distorted red blood cells filled the sinusoids. Erythrophagocytosis by the sinus endothelial cells was minimal. The follicles were inconspicuous and without germinal centers. The iron stain was strongly positive in all but one instance (case no. 1, Table II), in which the spleen was very small and in which the parenchyma was replaced by fibrous connective tissue.

Review of Cases from the Literature

A large number of cases of sickle cell disease have been reported but complete necropsy data are available in only a limited number. In reviewing the literature, only 13 cases were found in which there were included data pertaining to the age, sex, size of the spleen and liver, and the degree of erythrophagocytosis and hemosiderosis. These cases have been grouped according to the size of the spleens.

The first group consisted of 5 cases with normal or enlarged spleens (Table III). No average can be given since only one adult was included in this group, but the hepatic-splenic weight ratio clearly

indicates splenomegaly. Case no. 1, a colored girl, 4 years of age, was included because the weights of her spleen and liver were within normal limits.[11] The remainder of the cases showed a definite splenomegaly.

The second group consisted of 8 cases with siderofibrotic spleens and correspondingly high values for the hepatic-splenic ratios (Table IV). The age range in this group was from 6 to 38 years, the average age being 20.5 years. The spleens again were markedly atrophic

TABLE III

Collected Cases of Sickle Cell Disease with Spleens of Normal Weight, or Larger

Author and year	Age	Sex	Spleen gm.	Liver gm.	Liver-spleen ratio	Erythrophagocytosis		Hemosiderosis		Complicating disease
						Spleen	Liver	Spleen	Liver	
Wollstein and Kreidel,[5] 1928	4	F	30.0	460	15.3	—	++	++	+	
Wollstein and Kreidel,[5] 1928	3	M	210.0	460	2.2	—	++	+	+	
Wollstein and Kreidel,[5] 1928	3	M	182.0	430	2.4	—	++	+	+	
Lash,[14] 1934	21	F	960.0	2420	2.5	—	—	—	—	Cervical cesarean section
Ryerson and Terplan,[15] 1935	4	F	425.0	500	1.2	++	++	++	—	

(average 15.4 gm.). The livers were of normal size or enlarged (average 1834 gm.), as indicated by the uniformly high values for the liver-spleen ratios.

COMMENT

This survey indicates that varying degrees of erythrophagocytosis were present in sickle cell disease. The Kupffer cells of the liver most frequently exhibited engulfed red blood cells, while the splenic reticulum cells only occasionally showed signs of erythrophagocytosis. The changes in the spleen, however, were more varied in nature and more severe than those of the liver. An abnormal distention of the perifollicular sinusoids, hemorrhages and hemosiderosis constituted the different stages of the splenic lesion, producing the siderofibrotic spleen.

In the literature markedly enlarged spleens were reported chiefly in young children (Table III). There was only one adult in this group, a female, 21 years old. Cases with normal spleens, or with

spleens but slightly smaller than normal, were included in Table I. These probably are the less severe forms of sickle cell disease, as death in all instances was due to complicating conditions.

The cases tabulated in Tables II and IV are the severe forms of sickle cell disease with siderofibrotic spleens. Assembled respectively from the records of Charity Hospital of Louisiana and from the reported cases in the literature, these two groups show remarkable similarities.

TABLE IV

Collected Cases of Sickle Cell Disease with Small Spleens

Author and year	Age	Sex	Spleen gm.	Liver gm.	Liver-spleen ratio	Erythrophagocytosis		Hemosiderosis		Complicating disease
						Spleen	Liver	Spleen	Liver	
Graham,[16] 1924	30	F	28.0	2567	92.0	—	+	+++	+	Streptococcus septicemia
Sydenstricker,[12] 1924	6	M	7.9	690	87.0	—	—	+	—	
Jaffé,[17] 1927	8	M	10.0	900	90.0	—	++	++	—	Tuberculosis
Jaffé,[17] 1927	6	F	10.0	1300	130.0	—	++	++	+	
Ching and Diggs,[18] 1933	18	F	10.5	2210	210.0	—	+++	—	—	
Yater and Hansmann,[19] 1936	38	F	7.0	1715	245.0	—	—	+++	+	Abdominal crisis
Yater and Hansmann,[19] 1936	25	F	35.0	2090	59.7	—	—	+++	++	Abdominal crisis
Bauer,[20] 1940	33	F	15.0	3200	213.0	+	+	++	—	
Average	20.5		15.4	1834						

From the available clinical data it is difficult to establish the duration of active sickle cell disease. Diggs, [8] who studied thoroughly the changes in the spleen in sickle cell anemia, concluded that there is no direct correlation between the size of the spleen and the duration of the disease as indicated by the age of the patient. A large spleen was usually found in the early phases of the disease, while a small atrophic spleen was characteristic of the later phases. This is, however, not without exception. Therefore, the duration of the disease could be determined more accurately by the histologic changes present.

Cases included in Table I were characterized by normal or enlarged spleens, with histopathologic changes suggestive of shorter duration, and cases in Table II revealed siderofibrotic spleens, indicating longer duration. It is difficult to correlate these changes with the duration of the disease when one considers that the average age of the first group was 34.4 years and of the second, 19.0 years. There exist both active and latent varieties of sickle cell disease,[12] differing in degree rather than in kind. The first group probably represented the latent cases, and the second group the active cases. However, the pathologic changes differed only in degree. The enlarged spleens and the normal-sized spleens presented similar pathologic changes, differing again only in degree. In the active cases the liver-spleen ratios were much lower (Table III) or much higher (Tables II and IV) than normal, while in the latent form (Table I), they were nearly normal.

No direct correlation existed between erythrophagocytosis and complicating infectious disease [13] (Tables I to IV).

Summary

An example of sickle cell disease is reported in which there was a very marked degree of erythrophagocytosis.

Twelve cases of sickle cell disease were studied from 4,094 autopsies. These were divided into two groups: those with large and normal-sized spleens and normal or slightly enlarged livers; and those with siderofibrotic spleens and enlarged livers. These correlations were definitely demonstrated by the liver-spleen ratios.

Thirteen cases with complete necropsy data were collected from the literature and also tabulated according to the weight of their spleens. Definite splenomegaly was present in 4 cases. There was a normal-sized spleen in one case, and eight cases showed marked siderofibrotic changes with high values for the liver-spleen ratio.

The Kupffer cells were the most actively participating parts of the reticulo-endothelium in erythrophagocytosis. This process is not dependent upon coexisting infection.

The splenic changes may serve as criteria for the degree of activity but not for the duration of the sickle cell disease. The latent cases also present definite changes in the spleen and liver similar to those seen in active cases. The spleen may be enlarged, normal, or markedly fibrosed in sickle cell disease.

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[*Illustrations follow*]

DESCRIPTION OF PLATES

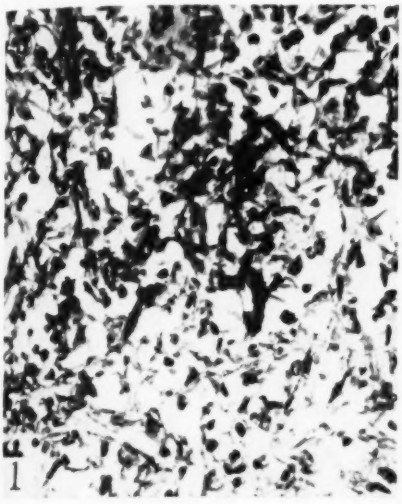
PLATE 27

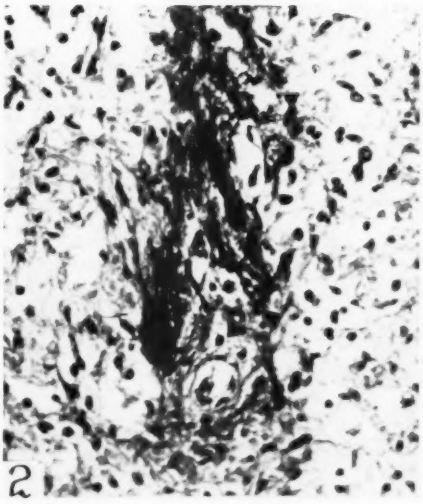
FIG. 1. (Case no. 4, Table II.) Spleen: marked distention of the sinusoids. Hemosiderin has been deposited in myceliumlike fashion. Turnbull-blue stain. × 300.

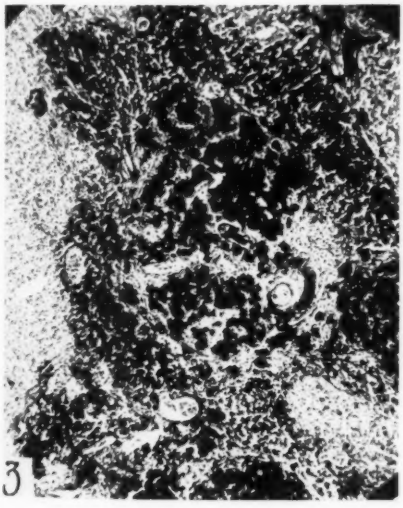
FIG. 2. (Case no 4, Table II.) Spleen: rodlike deposits of hemosiderin. Turnbull-blue stain. × 335.

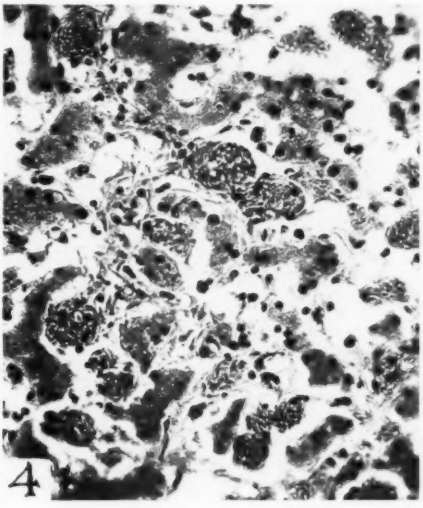
FIG. 3. Case no. 1, Table II.) Spleen: a typical siderofibrotic area with marked hemosiderin and calcium incrustation. Turnbull-blue stain. × 45.

FIG. 4. (Case no. 1, Table II.) Liver: the Kupffer cells show a marked degree of erythrophagocytosis. Hematoxylin and eosin stain. × 335.









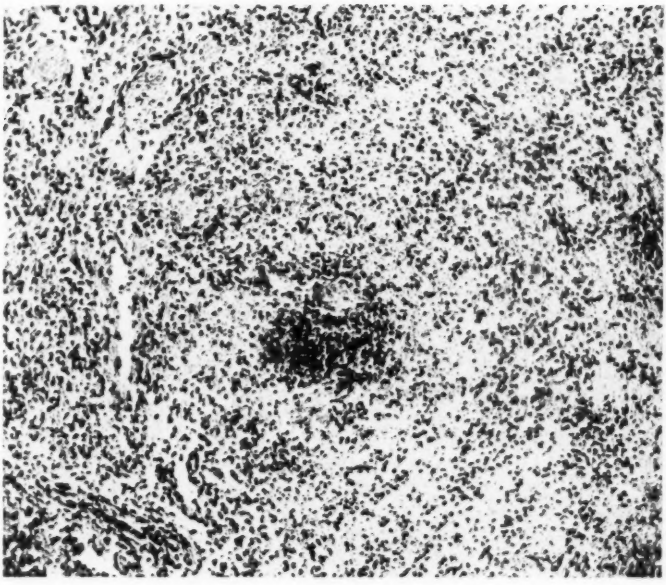
Stasney

Erythrophagocytosis in Sickle Cell Disease

PLATE 28

FIG. 5. (Case no. 7, Table I.) Spleen: markedly distended sinusoids. Sickle-shaped red blood cells are found in the pool around the lymph follicle. Hematoxylin and eosin stain. × 100.

FIG. 6. (Case no. 1, Table II.) Spleen and liver: marked disproportion in size.



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6

Stasney

Erythrophagocytosis in Sickle Cell Disease

HISTOLOGICAL CHANGES PRECEDING SPONTANEOUS LYMPHATIC LEUKEMIA IN MICE *

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According to its characteristic manifestations, leukemia is a generalized, or systemic, disease. However, the histogenesis of leukemia has been debated for many years and numerous theories have been presented. As Richter [1] pointed out in a recent review: "The various theories may be roughly grouped in two categories: according to one, the leukemic cells reach their unusual situations by metastases from their normal sites of origin. . . . ; according to the other, the cells are formed *in situ* where they are found." The observations herewith presented suggest that part of each of these categories may be correct, namely, that leukemia may develop by metastases following origin in abnormal sites.

Theories have been given free rein because material for the critical study of this question is rare. The absence of early clinical signs of leukemia has limited histological studies to relatively late stages, after the initial histogenesis has become confused, if not entirely hidden, by extensive proliferation.

Most of the discussions on the histogenesis of leukemia have been based on the examination of human material. Some of the more recent reviews and reports on leukemia and allied diseases have been presented, with consideration of current interpretations, by Richter,[1] Watson,[2] Klemperer,[3] Stasney and Downey [4] and Ehrlich and Gerber.[5] In the field of experimental leukemia the subject has been touched on only rarely. Hill [6] described various types of lymphoid hyperplasia in mice and concluded that leukemic cells were derived from the reticulum and that the first infiltrations occurred "in that portion of the mesentery binding the pancreas and the gut in region of the pylorus." Furth and Kahn,[7] from their work on transplantable leukemias, suggested that a malignant change in a single lymphocyte, rather than a systemic change, might be responsible for the origin of spontaneous leukemia. Barnes and Sisman [8] believed that their studies on malignant and nonmalignant myelosis did not answer the question concerning the origin of leukemic cells.

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Source of Materials Studied

The genetic aspects of the high incidence of spontaneous leukemia in mice of strain C58 have been discussed by MacDowell and Richter [9] and MacDowell.[10] Victor and Potter [11] have reported on the metabolic differences occurring in preleukemic mice of this same strain as compared with mice of the same age group from nonleukemic strains. Since 90 per cent of the mice of strain C58 that live longer than 6 months die with some form of leukemia, it was assumed that a fair proportion of these mice of leukemic age and still clinically normal would show, histologically, the significant early stages. Therefore 137 mice between the ages of 176 and 382 days, but clinically nonleukemic, were killed and the tissues from all organs saved. Most of the animals were obtained from retired mating pens and both sexes were represented, with females predominating (107 females to 30 males). The material was collected in 1933 and 1934, with additions in 1937 and 1938. The tissues from these preleukemic C58 mice were compared with tissues from a group of animals within the same age range from the nonleukemic strains, Storrs-Little and Bagg-albino, as well as with tissues from young C58 mice 6 to 8 weeks old.

All of the C58 and other strains of mice used in the present and preceding studies were bred and raised in the same colony under the same conditions of housing, diet and care.

A second source of material was a series of monthly biopsies performed by one of us (J. V.) for the study of the metabolic history of lymphatic tissues in individual mice during the period preceding leukemia. As planned, ten lymph nodes were to be removed singly from each of 24 mice of strain C58 at monthly intervals, beginning with animals 2 months old and omitting a biopsy at 3 months. At the end of this period, or when death occurred from any cause, all of the remaining tissues were to be examined microscopically. The method of biopsy, of course, provided only a small amount of isolated tissue at each period, the condition of which could not be compared with the animal's remaining tissues at the same time. The method, however, furnished helpful information on the probable sequence of events during the histogenesis of leukemia.

A third set of observations was made on tissues from 40 C58 and 10 Storrs-Little mice killed at intervals in connection with another experiment.

Tissues were fixed in Helly's or Zenker's fluids, with other methods being used for special purposes. Hematoxylin and eosin or hematoxylin and eosin with azur II were used for routine staining, but

other stains were used at times to accentuate certain histological or cytological characters. Flemming's fluid and triple staining were found useful for nuclear detail in small pieces of tissue.

Observations

In 59 of the 137 C58 mice examined *in toto*, reticulum cell hyperplasia was observed that had not been seen in young C58 mice or old mice of nonleukemic strains. In general the hyperplasia was confined to the reticulum of lymph nodes or to the perivascular regions of the livers. The spleen seemed to be involved in only 3 animals. The distribution of the hyperplasia was limited and in no case was there a generalized systemic reaction. When it occurred in lymph nodes, only one or two nodes were usually affected. In livers the lesions were not widely spread but were confined to a few foci. The hyperplasia occurred in both liver and lymph nodes in some cases, while in others only one or the other tissue was the seat of change. When a single node of a group was affected, *e.g.*, one of the cervical group, other nodes in the same group were usually unchanged. By designating a single node or liver tissue as a region, the 59 animals showed the following distribution: 1 region in 18 cases, 2 in 22 cases, 3 in 11 cases, 4 in 7 cases, and 5 in 1 case.

The 59 animals showing hyperplasia were of various ages and the changes appeared in both sexes (47 females and 12 males). These animals represented 44 per cent of the total females and 40 per cent of the total males examined. A description of the reticulum changes as they appeared in lymphoid and hepatic tissues follows.

Lymph Nodes

The earliest signs of reticulum hyperplasia in lymph nodes seemed to be confined to the medullary regions. In some cases only a small area was involved, while in others the reticulum cells displaced the remaining elements of the node to a great extent. Depletion of the free cells of the nodes, leaving the normal reticulum more apparent, could not account for the microscopical picture because of definite evidence of displacement of normal elements by the hyperplastic growth of the reticulum cells. It has not been possible to associate this reaction in C58 mice with any condition other than a susceptibility to spontaneous leukemia. Further evidence that this latter statement is valid was presented by MacDowell and Richter,[9] who showed that affections other than leukemia were rare in the 637 C58 mice examined by them at autopsy.

The reticulum cells were judged to be more compactly arranged than in normal regions, and there was no evidence that the hyperplasia originated in the follicles of the cortex. In extreme cases the normal architecture of a node was obliterated by the hyperplastic reticulum. This phenomenon was associated with a gross enlargement of the involved node in 9 cases.

There was no indication that the hyperplasia occurred in nodes of one region more often than in those of another. Similar histological pictures were seen in several instances in two nodes which were widely separated in the host's body. "Diffuse follicular hypertrophy" was not observed in any case during the preleukemic stages in C58 mice, although this condition has been said to be associated with the onset of leukemia.[2]

Liver

Simultaneously with, or independently of, changes in lymph nodes, reticulum hyperplasia was observed in the liver. The earliest stages of this proliferation appeared as small nodules of syncytial reticulum cells apparently originating from the perivascular reticulum. The extent of the proliferation varied from site to site and from animal to animal, but in no case was the hyperplasia general for all perivascular regions within a liver. Reticular fibers could be demonstrated in the lesions. In advanced stages, limits of the individual reticulum cells were more easily identified and various degrees of differentiation of cells of the lymphatic series were associated with the area of reticulum hyperplasia. Examples of this type of lesion from 4 different animals are shown in Figures 1–4. In terminal cases the participation of the reticulum was marked by progressive proliferation of lymphoid cells.

Spleen

Reticulum hyperplasia occurred in only three spleens. In these cases the affected areas were small and nodular, in close proximity to trabeculae but not associated with malpighian follicles. Perhaps because of the normally confused and shifting cellular population, histological and cytological changes in splenic tissue must be more pronounced in order to become assuredly apparent. Most certainly the point of origin of a single free cell in the red pulp of the spleen is a matter of conjecture, due to the continually changing topographical relationship of the free cells. It has been possible to assign only a minor part to the spleen in the origin of the leukemic state in C58 mice.

Other Tissues

All lesions in the remaining tissues were infiltrative in character, with no evidence of local origin from fixed tissues. After proliferation of malignant free cells began at any site, transport and invasion became widespread and could account for most of the lesions found in terminal cases. It is impossible to determine from these studies on our material how long the preleukemic stages exist before the disease becomes clinically apparent, but it seems obvious that there would be considerable variation in this respect.

Response of Preleukemic Lesions to Carmine Injections

As a possible means of obtaining a measure of the functional state of the reticulum cells in the preleukemic lesions, a group of 11 animals of leukemic age were given injections of carmine. Of this group, 5 animals were found to have either lymph node or liver changes, or both, of the desired types. The amount of carmine injected was sufficient for general distribution throughout the animal, but in no case was there a concentration of the dye within the areas of reticulum hyperplasia described above. However, the more clearly differentiated cells, such as the Kupffer cells of the liver, known to be phagocytic, were loaded with particles of dye and were often in close proximity to the hyperplastic areas. We interpret this result as showing that the stimulated reticulum in the preleukemic lesions of C58 mice did not have histiocytic tendencies under the existing conditions. This evidence is considered of importance because of the general acceptance of the lymphopoietic potency of nonstoring cells of the reticulum.

Biopsy Experiments

Instead of making available all the tissues from one animal at a given moment, the biopsy experiments gave samples of lymphatic tissue from the same animal at regular intervals. Such successive samples revealed directly the relationship between reticulum hyperplasia and lymphopoiesis on the one hand and leukemia on the other.

The results of histological analysis of the individual nodes at biopsy and of all tissues at autopsy are given in Table I. The animals are grouped according to the terminal diagnosis; those which died early either without leukemia or undiagnosable on account of postmortem change are given, but do not contribute directly to answering the question at hand.

The significant point is that the 10 cases of leukemia at autopsy were preceded without exception by reticulum hyperplasia and

TABLE I

Histological Condition of Individual Lymph Nodes at Biopsy

Animal no.		Diagnosis based on all tissues at autopsy																						
		Leukemic										Not leukemic							Early death without diagnosable leukemia						
		6	8	10	13	14	15	17	18	19	20	3	4	5	12	16	21	22	1	2	7	9	11	23	24
Lymph node	Age mos.																								
1st	2	N	N	N	N	N	N	N	N	N	N	N	—	N	—	N	N	N	N	N	N	N	N	N	N
2nd	4	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3rd	5	—	hd?	h?	—	N	N	N	N	—	N	N	N	h?	N	—	h?	N	N	N	hd?	N	N	—	N
4th	6	h?	hd	hd	—	N	—	—	h	—	h	N	N	N	N	N	—	N	N	N	—	N	—	—	N
5th	7	—	N	—	hd	N	N	—	N	h	hd+	N	N	h?	N	N	—	N	N	N	hd	N	N	—	N
6th	8	h	—	hd	hd	hd	—	hd	N	hd	L	N	N	—	h	—	—	N			*		—	N	N
7th	9	hd	—		L	h	h	h	N	L		N	N	—	hd	N	—	h?					h		
8th	10	—	—			hd	hd	—	N			N	N	h	N	N	—	hd?					—		
9th	11	hd	L				L	hd	+hd+			N	h	h	hd	hd	hd	h?					hd		
10th	12	hd	L				L	L	hd			N	hd	hd	hd	hd	hd	hd?					*		

N = Normal
h = reticulum hyperplasia
d = lymphopoiesis
+ = marked

L = histological leukemia
— = insufficient tissue obtained
* = post mortem changes precluded diagnosis

lymphopoiesis. Five of these mice died with leukemia before the end of the experiment and 5 showed leukemia histologically when killed. That similar reticulum hyperplasia occurred in 6 cases without leukemia at autopsy is not significant, since in each of these cases the outcome was concealed by the arbitrary killing after the tenth node was removed.

Animal no. 20 gave the most orderly sequence of stages approaching leukemia; photomicrographs of sections from these tissues are shown in Figures 5–7. However, the frequent failure of successive nodes from the same animal to show a regular progression (7 cases) is evidence that the initial stages do not occur uniformly throughout the lymphoid system. In 2 cases (animals nos. 6 and 18) the tenth node did not show leukemia, but other tissues did. With such irregularity in the distribution of the early manifestations, it is entirely possible that the removal of an especially active node may have altered the course of events.

These observations on tissues from the same animal covering a period of 10 months confirm the conclusion that reticulum hyperplasia precedes the spontaneous production of leukemia cells.

Miscellaneous Series

In connection with another experiment further evidence was obtained showing that reticular changes are typical of strain C58 mice after the age for spontaneous leukemia is reached. From a group of 40 C58 and 10 Storrs-Little (a nonleukemic strain) mice of the same age, individuals were killed at intervals and examined histologically. Of the 24 C58 mice killed before 6 months of age none showed reticular hyperplasia; of the 16 killed at 6 months, 7 showed reticular hyperplasia identical with that reported above in either lymph nodes or liver. The Storrs-Little animals showed no reticular hyperplasia at any time (7 killed before and 3 killed at 6 months).

Discussion

To demonstrate conclusively the formation of one type of cell from another type by present histological methods it is necessary to have a picture of progressive zonal development, as for example in the formation of spermatozoa. With the lack of delimited areas of progressive differentiation it is usual to construct a closely knit series dependent on overlapping morphological criteria. This latter method has been the one used in the studies on blood cell formation, and the subjective reactions involved may perhaps explain in part the long-drawn-out controversy on the subject. However, there is almost unan-

imous agreement among present-day investigators that lymphocytes may be derived from undifferentiated, nonstoring multipotent cells of the reticulum, a topic recently reviewed by Bloom.[12]

In the absence of a generalized hyperplasia of lymphoid tissues during the preleukemic period in C58 mice, the localized reticulum hyperplasia (absent in nonleukemic strains) and associated lymphopoiesis become important in considering the source of leukemic cells. In concluding that the immature lymphocytes which appear in the areas of reticulum hyperplasia originated from the reticulum cells, the morphological method was used and the possibilities of subjective error are admitted. However, three observations can hardly be questioned: the failure of immature cells to leave lymph nodes during the preleukemic period, the inactive state of germinal centers in lymph nodes, and the presence of cell intergradations from reticulum cell to lymphocyte in the regions designated.

Accepting the reticulum lesions described here as sites for primary leukemic cell production of a focal nature, the widespread terminal infiltrations may readily be explained. After the formation of lymphocytes with a leukemic constitution, the motility of the lymphocyte and the ease with which it is transported within the lymphatics and the blood stream offer acceptable explanations for the formation of metastases which could account for the systemic infiltrations in terminal cases. Further support for belief in this metastatic type of lesion comes from the studies on the characteristics of leukemic cells during transfer.

Transplantation of experimental leukemias has produced strong evidence that each leukemic cell population is autonomous and that the final distribution of lesions depends on the site of entry, the intrinsic characteristics of the particular transplanted leukemic cells studied and the interaction between the host and the cells injected.[13-15] Assuming that populations of leukemic cells arising spontaneously have similar intrinsic characters which determine the tissues which they will infiltrate, the point of origin of cells studied in terminal lesions of a spontaneous case becomes a matter of conjecture. Since Hill's[6] statements concerning the histogenesis of leukemia and allied diseases in her strain of mice were based upon observations on terminal cases, they can have little significance in interpreting early stages.

As pointed out before, in the early stages of spontaneous leukemia, when the number of lesions is small, two types of lesions are regularly found: one with reticular activity associated with the production of lymphocytes, and the other with characteristics of the metastatic type

found in transplanted leukemias. In the terminal phase evidence of participation by the reticulum disappears.

Although we have found no evidence of participation by the normally cytopoietic reticulum in germinal centers of lymph nodes in the development of spontaneous leukemia in C58 mice, Ehrlich and Gerber [5] found such evidence in the development of lymphocytic neoplasia in human material. Their evidence for progression from a lesion of reticular type to a lymphocytic type is clear. From a theoretical standpoint it is possible for any lymphopoietic tissue to respond to the unknown stimuli which result in the production of leukemia and allied diseases. The important part that the genetic constitution plays in the determination of neoplasia has been clearly demonstrated. The consistent preleukemic changes in C58 mice may be due to the genetic constitution of the strain, and with the same stimulant against other genetic backgrounds a different, but perhaps just as consistent, picture might be obtained.

The multipotency of the reticulum cell precludes the possibility of identifying leukemic progression by the mere recognition of reticulum hyperplasia without observations of later stages. Klemperer [3] has warned of such misinterpretation, and has shown the importance of the reticulum in diseases of the hemopoietic system, as have Stasney and Downey [4] and others.

A few authors have questioned the conception of leukemia as a disease of systemic origin. In a review, Watson [2] called attention to "the unquestioned existence of leukemia in which the anatomic lesions are well localized, with minimal or no changes elsewhere." This observation points toward a restricted origin of primary malignant change. Furth and Kahn,[7] having concluded that leukemia in mice may be transmitted by a single cell, suggested that a malignant change in a single lymphocyte may be the origin of spontaneous leukemia. This hypothesis would eliminate the participation of fixed lymphopoietic tissues in the malignant change but would most certainly localize the origin of the disease. In connection with a discussion of the origin of leukemic cells, Furth [16] also has pointed out that histological methods may lead to error in interpretation, and that the tissue culture technic has been of little value in the study of histogenesis of blood cells from fixed tissues. We agree that the final answer to the question of the spontaneous source of leukemic cells, and many normal cells for that matter, will have to wait until existing technics are perfected or new ones are discovered. However, at this time the great mass of evidence points to a direct relationship between the reticulum and the formation

of blood cells under normal and pathological conditions. The correlation between reticulum cell hyperplasia, abnormal lymphopoiesis and the onset of leukemia in C58 mice makes it necessary for us to conclude for the present that the spontaneous production of leukemic cells is the result of stimulation of fixed lymphopoietic tissues.

Summary

We interpret our observations on tissues from preleukemic mice of strain C58 as showing that in this particular strain restricted areas of reticulum hyperplasia become the sites for primary malignant lymphocytopoiesis. These areas occur particularly in the medullary tissue of lymph nodes and in perivascular regions of the liver. Such areas have not been observed in nonleukemic strains. In mice of strain C58 hyperplasia of the germinal centers of lymphoid tissues was not observed in early stages of leukemia. Following the production of a population of free malignant lymphocytes, invasion accounts for the majority of the widespread lesions common to the terminal stages of leukemia. The concept of a widespread systemic origin of leukemia may have been due in part to the relatively late stage of the disease at which tissues have previously been examined.

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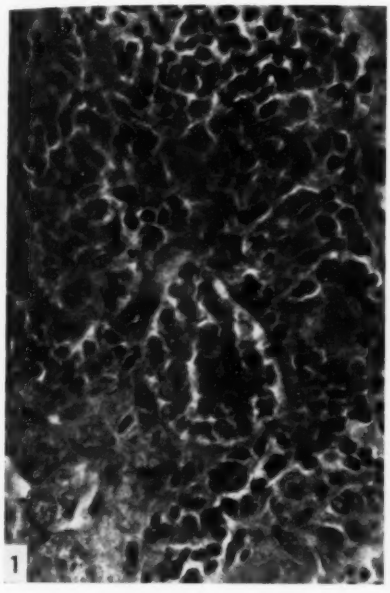
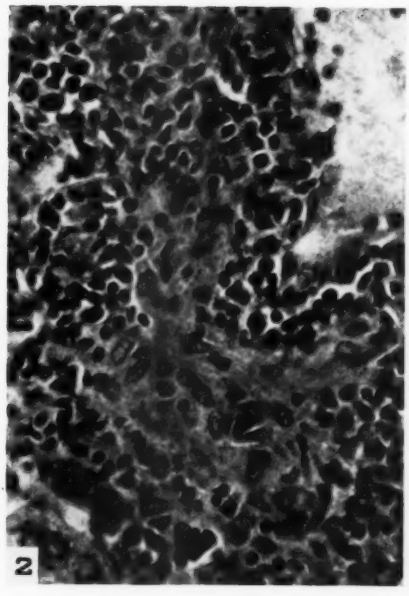
[*Illustrations follow*]

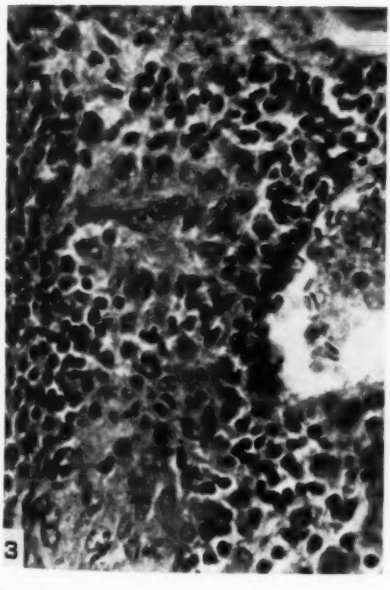
DESCRIPTION OF PLATES

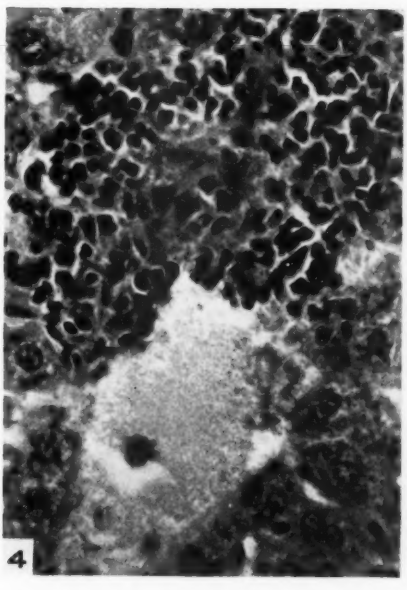
PLATE 29

FIGS. 1–4. Focal areas of periportal lymphopoiesis in the livers of four preleukemic C58 mice. × 460.









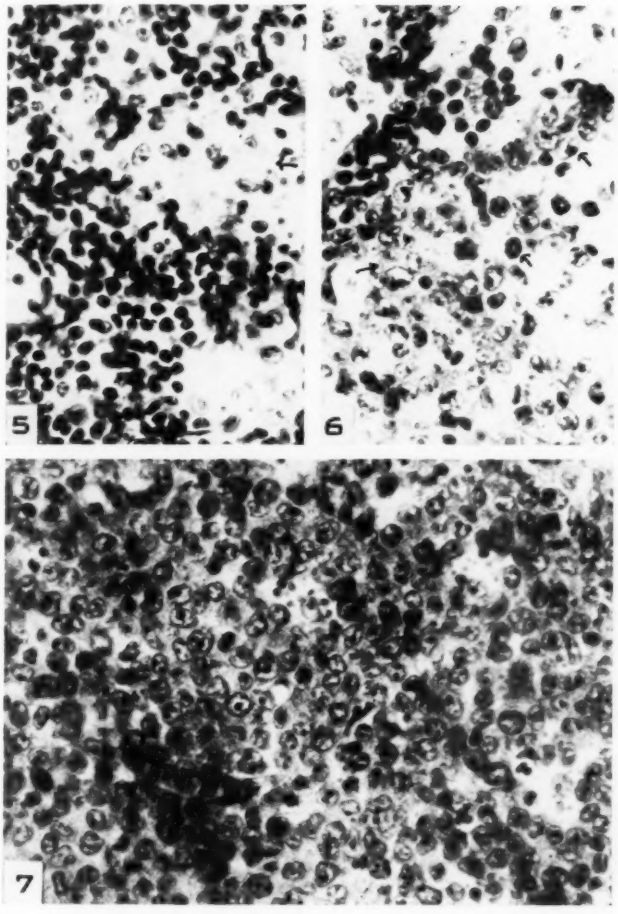
Potter, Victor and Ward Changes Preceding Lymphatic Leukemia

PLATE 30

FIG. 5. Section of medullary tissue of lymph node removed from animal no. 20 (6 months old) at the fourth biopsy, showing reticulum cell hyperplasia and displacement of normal elements. × 460.

FIG. 6. Section of medullary tissue of lymph node removed at the fifth biopsy from animal no. 20 (7 months old) showing reticulum cell hyperplasia and lymphocytopoiesis. × 460.

FIG. 7. Leukemic lymph node removed from animal no. 20 (8 months old) at the sixth biopsy. × 460.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6
7

IN VIVO NEUTRALIZATION OF PERTUSSIS TOXIN WITH PERTUSSIS ANTITOXIN *

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An interstitial mononuclear pneumonia similar to that occurring in pertussis has been produced in rabbits by intratracheal injection of cultures of the Bordet-Gengou bacillus,[1] by certain bacterial toxins,[2] and by viruses.[3] Gallavan and Goodpasture [4] showed that the Bordet-Gengou bacilli could be demonstrated only on the cilia of the bronchi and bronchioles and not in the lung tissue. Similarly, in chicken embryos they could demonstrate the Bordet-Gengou organisms only on the cilia of the bronchi, bronchioles and esophagus.

These findings suggested that the mononuclear reaction in the lungs could be the result of the action of some toxic substance produced by the bacilli in the bronchi and diffusing into the surrounding tissues. The test of this hypothesis was not possible, however, until a toxic substance capable of producing such a lesion could be isolated from pertussis organisms. Recently, Roberts and Ospeck [5] reported the preparation of such a substance. This material is a heat-labile toxin obtained from cultures of the intermediate phases of the Bordet-Gengou bacillus. It will kill mice and produce necrosis in rabbit's skin. An antitoxin capable of neutralizing the action of this toxin was also described by these authors.

It was thought desirable (1) to determine if intratracheal injection of this toxin in rabbits could produce an interstitial mononuclear reaction similar to that occurring in pertussis, (2) to see if administration of antitoxin could neutralize or modify the lesion, and (3) to observe the effect of antitoxin administration on the lesions produced by injection of freshly isolated phase I Bordet-Gengou organisms. Regardless of whether pertussis in man results from infection by the Bordet-Gengou organisms alone, or represents the action of a combination of these organisms and a virus, it is generally recognized that the extensive damage to the lungs and bronchi is dependent upon the presence and multiplication of these bacteria. Hence, the demonstration of any inhibiting effect of antitoxin upon the mononuclear reaction in the lungs produced by these bacteria would be important in the control of this disease.

* Received for publication, June 10, 1942.

Methods and Materials

Animals. Normal, adult, male, white rabbits weighing about 2 Kg. were used.

Toxin. The toxin employed was prepared from a phase II Bordet-Gengou bacillus and sent to us by Dr. M. E. Roberts of the Lederle Laboratories. The titration values of the toxin and antitoxin were determined by Dr. Roberts, who defined as a toxic unit (M. L. D.) the amount necessary to kill 50 per cent of mice 4 days after intravenous inoculation. This mouse unit is approximately 40 times the amount necessary to produce a skin lesion in the rabbit. Several batches of toxin with different titers were used, but in all cases 2 cc. amounts were injected directly into the trachea, after making an aseptic incision in the skin of the neck. Two cc. amounts were used as it was found that this amount of heat-inactivated toxin caused no significant pathologic changes in the lung.

Antitoxin. The antitoxin, made by the prolonged injection of the toxin into rabbits, also was supplied to us by Dr. Roberts. The unit of antitoxin as determined by Dr. Roberts was defined as that amount of antitoxin which would neutralize one M. L. D. of toxin. In our experiments the antitoxin was injected intravenously 24 hours before the intratracheal injection of toxin. The antitoxin, diluted 1 to 80, weakly agglutinated suspensions made of phase I, II, and III Bordet-Gengou organisms as well as a suspension of parapertussis bacilli.

Organisms. The Bordet-Gengou bacilli employed in these experiments were freshly isolated strains kindly supplied by Dr. J. G. M. Bullowa. The organisms were grown for 48 hours on a medium similar to that described by Bordet and Gengou [6] except for the substitution of 20 per cent defibrinated human blood for horse blood. The growth on a large number of slants was washed off in sterile physiologic saline solution, pooled, rapidly frozen, lyophilized and sealed in a vacuum. This treatment was found not to damage the organisms. The approximate number of organisms injected was 100 billion (2 slants), although in some experiments 8 slants or about 400 billion organisms were used.

Necropsy. The animals dying within 4 days were necropsied shortly after death. The remainder were killed 96 hours after the toxin was injected, and necropsied immediately. The lungs were removed with the trachea, gently inflated with air and fixed in Zenker's solution. The sections were embedded in paraffin and stained with hematoxylin and eosin, and for bacteria according to MacCallum's method.[7]

Experimental

Toxin-Antitoxin Experiment

This experiment was designed to study the effect of intratracheal injection of the toxin on the rabbit's lung and the ability of the antitoxin to neutralize the toxin. Thirty-two rabbits were injected intratracheally with various amounts of toxin. Fifteen of these had received various doses of antitoxin intravenously 24 hours previously. The results of these experiments are shown in Table I. Of 17 rabbits receiving no antitoxin, 10 died within 72 hours after injection and 5 of the remaining 7 had a reaction which we have described below as 4 plus. Those which died within 24 hours showed extensive edema. The others had necrosis and cellular proliferation similar to that which we have described as a 4 plus reaction. In these lungs there were large areas in which the alveoli and the alveolar walls were filled with large

Table I

Results Obtained in the Toxin-Antitoxin Experiment

No. of rabbit	Amount of antitoxin *cc.*	Strength of antitoxin *units*	Total no. of units	Amount of toxin *cc.*	Strength of toxin *units*	Total no. of units	Type of lesion
3	0	—	—	2	100	200	++++
4	0	—	—	2	100	200	++++
7	0	—	—	2	100	200	+++
8	0	—	—	2	100	200	++
22	0	—	—	2	100	200	++++
23	0	—	—	2	100	200	++++
24	0	—	—	2	100	200	Died 72 hr.
20	0	—	—	2	150	300	++++
21	0	—	—	2	150	300	Died 24 hr.
13	0	—	—	2	750	1,500	Died 72 hr.
14	0	—	—	2	750	1,500	Died 24 hr.
15	0	—	—	2	750	1,500	Died 24 hr.
16	0	—	—	2	750	1,500	Died 48 hr.
27	0	—	—	2	750	1,500	Died 24 hr.
28	0	—	—	2	750	1,500	Died 24 hr.
29	0	—	—	2	750	1,500	Died 48 hr.
30	0	—	—	2	750	1,500	Died 72 hr.
19	5	7,000 per cc.	35,000	2	100	200	+++
18	5	7,000 per cc.	35,000	2	163	326	+
17	5	7,000 per cc.	35,000	2	163	326	+
5	5	9,000 per cc.	45,000	2	100	200	+++
6	5	9,000 per cc.	45,000	2	100	200	+
25	7	7,000 per cc.	49,000	2	750	1,500	++
26	7	7,000 per cc.	49,000	2	750	1,500	++
31	7	7,000 per cc.	49,000	2	750	1,500	+
32	7	7,000 per cc.	49,000	2	750	1,500	++
1	6	9,000 per cc.	54,000	2	100	200	+
2	6	9,000 per cc.	54,000	2	100	200	+
9	15	7,000 per cc.	105,000	2	750	1,500	+++
10	15	7,000 per cc.	105,000	2	750	1,500	Died 24 hr.
11	15	7,000 per cc.	105,000	2	750	1,500	+
12	15	7,000 per cc.	105,000	2	750	1,500	+++

mononuclear cells. These cells are thought to be both macrophages and alveolar epithelial lining cells in view of the work of Ross,[8] who showed by supravital staining methods that the large mononuclear cells occurring in the alveoli of rabbits injected with staphylococcic toxin were both macrophages and epithelial lining cells. Around the blood vessels and bronchi were large numbers of lymphocytes. The blood vessels showed scattered areas of extensive endothelial hyperplasia. In other areas of the lung were large areas of necrosis (Fig. 1), some of which were infiltrated by numerous polymorphonuclear leukocytes. There were also areas in which hemorrhage and edema were prominent features. The bronchioles frequently showed some cellular infiltration and focal necrosis of the epithelium. One animal had a 3 plus reaction (Fig. 1) with changes similar to those described as 4 plus except for the absence of marked necrosis. The changes in the remaining rabbit were classified as a 2 plus reaction. The alveolar walls were thickened by large mononuclear cells. The perivascular lymphatics and the peribronchial tissues contained large numbers of lymphocytes. An occasional bronchiole showed a few round cells in the epithelial layer.

Of the 15 rabbits previously prepared with antitoxin, 7 showed a 1 plus reaction. A few macrophages were seen in both the interstitial tissue and the alveoli, and a moderate number of lymphocytes were found around the blood vessels and bronchi. Three rabbits had a 2 plus reaction, 4 had a 3 plus reaction, and 1 died with pulmonary edema within 24 hours after inoculation of the toxin.

From these results it is obvious that the toxin can produce results similar to those occurring in pertussis. The antitoxin, although it did not completely prevent the lesions, materially reduced them. The rabbits receiving large doses of antitoxin are discussed below.

Bordet-Gengou Organism-Antitoxin Experiments

Experiments were designed to determine whether the antitoxin would prevent or modify the lesions produced by the injection of freshly isolated Bordet-Gengou bacilli. Thirty-eight rabbits were used. The results and dosages are shown in Table II. Twenty rabbits received the antitoxin intravenously 24 hours before the organisms were injected. Of the 20 animals which received both organisms and antitoxin, 17 had only a 1 plus reaction and 3, a 2 plus reaction. Eighteen rabbits received the organisms alone: 2 of these died within 72 hours of injection, 2 had a 4 plus reaction, 9 had a 3 plus reaction, 4 had a 2 plus reaction and 1 had a 1 plus reaction.

There was no evidence of multiplication of the Bordet-Gengou

bacilli in the lungs. Hence it is thought that the lesions were the result of toxin formed by the disintegration of the organisms injected.

TABLE II

Results Obtained in the Bordet-Gengou-Antitoxin Experiment

No. of rabbit	Amount of antitoxin *cc.*	Strength of antitoxin *units*	Total no. of units	No. of organisms *slants*	Type of lesion
4	0	—	—	2	++
5	0	—	—	2	+++
6	0	—	—	2	+++
15	0	—	—	2	+++
16	0	—	—	2	+
13	0	—	—	4	+++
14	0	—	—	4	+++
19	0	—	—	4	++
20	0	—	—	4	+++
28	0	—	—	4	+++
29	0	—	—	4	+++
30	0	—	—	4	++++
33	0	—	—	4	++++
34	0	—	—	4	Died 72 hr.
35	0	—	—	4	Died 72 hr.
36	0	—	—	4	++
17	0	—	—	8	++
18	0	—	—	8	+++
12	6	6,000 per cc.	36,000	2	+
9	7	6,000 per cc.	42,000	4	+
10	7	6,000 per cc.	42,000	4	+
11	7	6,000 per cc.	42,000	2	+
8	8	6,000 per cc.	48,000	2	+
31	7	7,000 per cc.	49,000	4	+
32	7	7,000 per cc.	49,000	4	+
23	6	9,000 per cc.	—	4	+
1	10	6,000 per cc.	60,000	2	+
2	10	6,000 per cc.	60,000	2	+
3	10	6,000 per cc.	60,000	2	++
7	10	6,000 per cc.	60,000	2	+
21	7	9,000 per cc.	63,000	8	+
22	7	9,000 per cc.	63,000	8	++
24	7	9,000 per cc.	63,000	4	+
37	7	9,000 per cc.	63,000	4	+
38	7	9,000 per cc.	63,000	4	+
25	10	9,000 per cc.	90,000	4	+
26	10	9,000 per cc.	90,000	4	+
27	10	9,000 per cc.	90,000	4	++

From these experiments it is seen that the antitoxin neutralized much of the toxin.

The results of both experiments are summarized in Table III.

DISCUSSION

The pathogenesis of pertussis in man is an exceedingly complex problem. In the experiments cited here, as in our previous experiments,[1] the conditions cannot be compared to those found in the course of natural infection by the Bordet-Gengou organisms. In none of our previous experiments were we able to culture the organisms from the lungs of rabbits inoculated with large doses of Bordet-Gengou bacilli.

It was, therefore, our belief that the interstitial mononuclear reaction resulted from the action of some toxic substance released by the disintegration of the inoculated bacteria. If this were true, then it was not necessary to assume that a virus infection was present since it had been shown that bacterial toxins can produce a lesion identical to that caused by viruses.

The experiments presented in this paper further strengthen this thesis since it was shown that an interstitial mononuclear pneumonia

TABLE III

Summary of All Experimental Data

	Small doses					Large doses				
	Toxin (200–326 M. L. D.)					Toxin (1500 M. L. D.)				
	Died	++++	+++	++	+	Died	++++	+++	++	+
No antitoxin	2	5	1	1	—	8	—	—	—	—
Antitoxin 35,000–54,000 units	—	—	2	—	5	—	—	—	3	1
Antitoxin 105,000 units	—	—	—	—	—	1	—	2	—	1
	Bacteria (2 slants)					Bacteria (4–8 slants)				
No antitoxin	—	—	3	1	1	2	2	6	3	—
Antitoxin 36,000–49,000 units	—	—	—	—	3	—	—	—	—	4
Antitoxin 54,000–63,000 units	—	—	—	1	3	—	—	—	1	5
Antitoxin 90,000 units	—	—	—	—	—	—	—	—	1	2

can be produced by a cell-free, heat-labile substance isolated from Bordet-Gengou bacilli. The extent of this lesion also can be reduced by the previous injection of an antitoxin prepared from this material. Furthermore, the lesions caused by the injection of freshly isolated Bordet-Gengou organisms in phase I also are markedly reduced, if antitoxin is administered prior to the organisms.

The data summarized in Table III illustrate another interesting point, namely, that with large doses of antitoxin an occasional rabbit may give a more severe reaction when toxin is injected than if a smaller dose of antitoxin were given. Thus 1 rabbit died and 2 had a 3 plus reaction when injected with 1,500 M. L. D. of toxin after receiving 105,000 units of antitoxin. In contrast, of 4 rabbits receiving approximately half that amount of antitoxin, none gave more than a 2 plus reaction to the same dose of toxin. These findings suggest that the

lesions in the former group may have been caused in part by an allergic reaction due to passive sensitization to some other antigenic substance present in the toxin. No such reaction was observed in animals injected with Bordet-Gengou bacilli, presumably because the release of the material from the organisms was slower and the concentration less.

The results of these experiments cannot be applied directly to the problem of pertussis in man, since the organisms injected intratracheally in the rabbit do not multiply but rapidly disintegrate, thus releasing a large amount of toxin. In man, however, as Gallavan and Goodpasture [4] have shown, the organisms multiply on the cilia of the bronchi and bronchioles. They disintegrate over a period of time, thus providing a steady supply of toxin to the lung over longer periods than in the rabbit. Hence, it is thought that the experiments in the rabbit were a more severe test of the neutralizing power of the antitoxin than would have been encountered had there been available an animal in which the organism could multiply. Of course, it will be necessary to test this antitoxin in the presence of the naturally occurring disease in man, but to us there seems to be every reason to expect that the lesions produced by the organisms will be inhibited by this antitoxin.

Summary

Experiments are reported in which it is shown that a heat-labile toxin obtained from cultures of the intermediate phase of the Bordet-Gengou bacillus can produce an interstitial mononuclear reaction similar to that caused by the Bordet-Gengou bacillus.

It is also shown that an antitoxin prepared from this toxin, when injected prior to either the toxin or the Bordet-Gengou organisms, is capable of greatly modifying the extent of the lesion produced.

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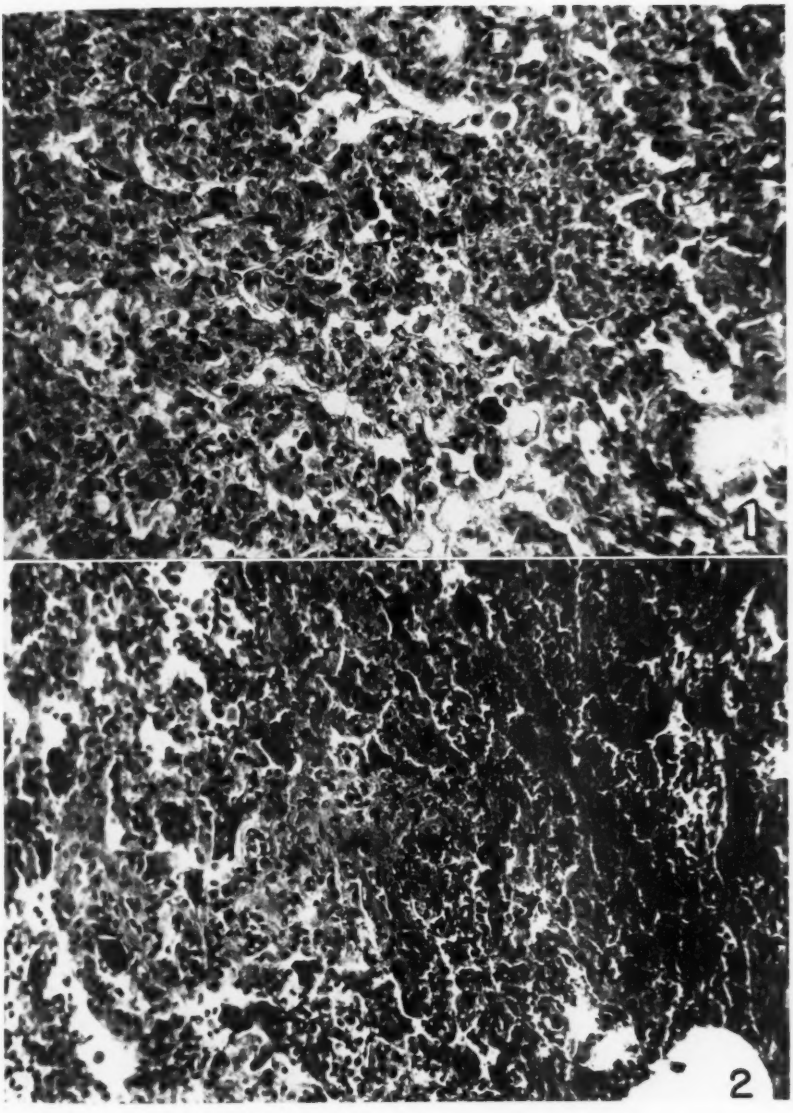
DESCRIPTION OF PLATES

PLATE 31

FIG. 1. Section of the lung of a rabbit receiving pertussis toxin but no antitoxin. This animal was killed 96 hours after injection of the toxin. This reaction was listed as a 3 plus reaction and shows marked proliferation of mononuclear elements. Hematoxylin and eosin stain. × 200.

FIG. 2. Section of the lung of a rabbit receiving pertussis toxin but no antitoxin. This reaction was listed as a 4 plus reaction. An area of necrosis may be seen. Hematoxylin and eosin stain. × 400.



Sprunt and Martin Neutralization of Pertussis Toxin

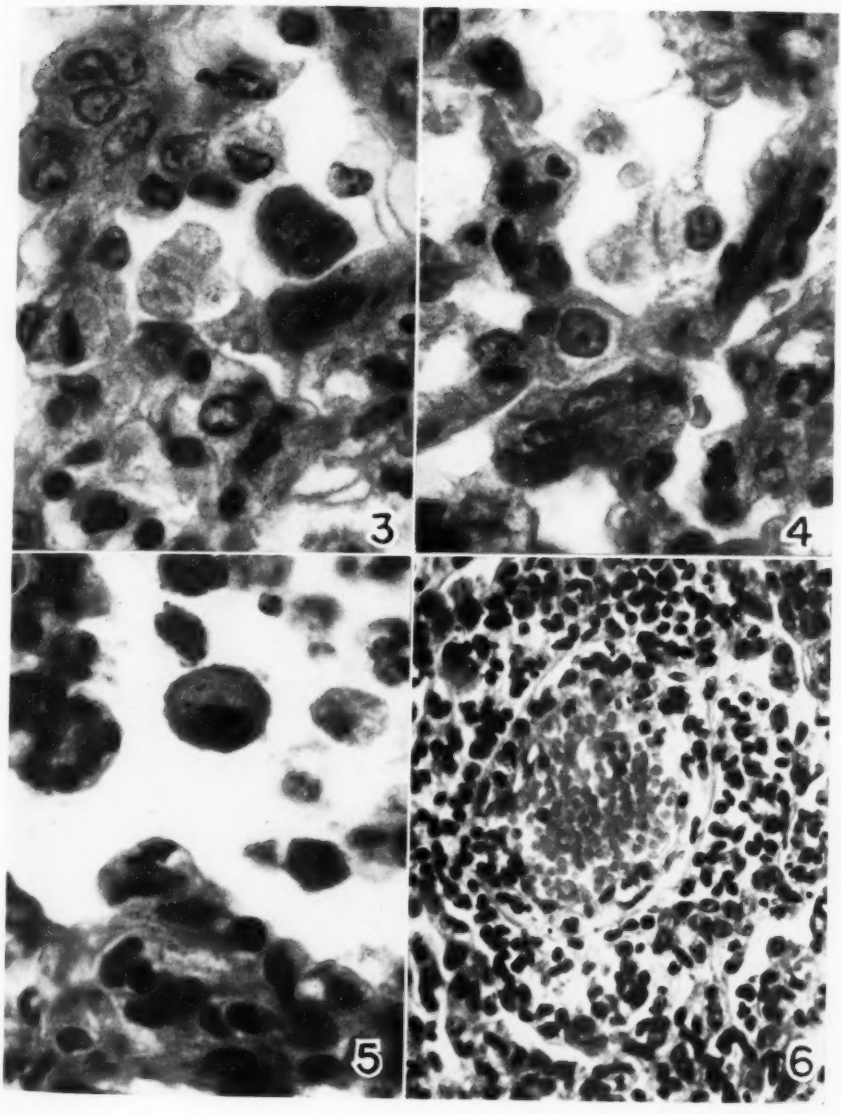
PLATE 32

All figures were taken from the same section as Figure 1.

FIGS. 3 and 4. These areas show thickening of the alveolar walls and mononuclear cells in the lumina. Hematoxylin and eosin stain. × 900.

FIG. 5. In this area there are macrophages in an alveolus. Hematoxylin and eosin stain. × 900.

FIG. 6. A perivascular infiltration of lymphocytes is shown in this area. Hematoxylin and eosin stain. × 400.



Sprunt and Martin Neutralization of Pertussis Toxin

THE EFFECT OF CRYSTALLIZED BOVINE SERUM ALBUMIN ON THE TISSUES OF NORMAL ANIMALS

I. Morphologic Changes in Normal Rabbits Induced by Intravenous Injection of Crystallized Bovine Serum Albumin*

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The methods for the fractionation of plasma proteins recently developed in the Department of Physical Chemistry of the Harvard Medical School have made available the proteins of blood plasma in states of great purity and in far larger amounts than could readily have been yielded by previous methods. The use of these materials has provided a new approach to many problems in biology and in clinical medicine.[1-8]

Of the proteins prepared by the new technic, the albumin of human plasma has been made available in considerable amounts by Cohn, Strong, Oncley and Armstrong, and has already been used in the clinic, especially in the treatment of shock.[3, 5-8] Because of the necessity of obtaining large quantities of material for use in the treatment of shock, a substitute for human serum albumin is required. Cohn and Hughes have prepared crystallized albumin from bovine plasma.

Before using bovine serum albumin as a therapeutic agent in man, it seemed advisable to determine its effect on animals. Biologic agents produced from one species of animals may lead to different results when injected into heterologous animals of different species. In spite of this, the effects of bovine serum albumin on experimental animals may serve as a useful guide in determining the suitability of this material for human therapy. The present report is devoted to a study of the consequences of intravenous injection of bovine serum albumin in the rabbit.

Bovine serum albumin injected in various species of animals and in man is not excreted as such by the kidneys. Since the albumin does not appear in the urine and no other method of excretion has yet been

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found, it is presumed to be dealt with by the organism receiving the injection. The albumin might be used in metabolism, or it might be stored, with or without chemical modification. It might or might not produce, in various organs, changes which would serve as contraindications for its use therapeutically. These problems can best be studied in normal animals, in which tissue alterations not due to the albumin are at a minimum.

Rabbits were chosen as the first experimental animals to be studied because of the ease of intravenous injection, because they belong to an entirely different species than that from which the albumin was obtained and because the normal variations in histology are well known. Other species of animals have been used, with results for the most part similar to those in rabbits. These experiments will be discussed in subsequent reports.

The experiments with crystallized bovine serum albumin have been designed to determine the effect of a single intravenous injection of a size comparable, per Kg. of body weight, to that used in man; of repeating this injection once, and of repeating the injection several times.

Materials and Methods

Properties of the Crystallized Bovine Serum Albumin

Crystallized bovine serum albumin in the solid state is a white powder composed of crystals of various shapes. The molecular weight as determined by osmotic pressure is 70,000. A comparable figure for the molecular weight has been obtained by using the ultracentrifuge. The impurities detected by the methods available are less than 0.5 per cent. The crystallized bovine albumin under consideration is readily soluble in water, yielding clear solutions even at concentrations greater than that used in the animal experiments (25 per cent).

For the purposes of the experiments described in this report, 25 gm. of the crystallized bovine serum albumin was dissolved in 100 cc. of water especially distilled for intravenous use. The solution was passed through a Seitz filter (D-2 pad). Sodium bicarbonate (0.375 gm. per 100 cc. solution) and sodium chloride (0.55 gm. per 100 cc. solution) were added. The solution was then sterilized by filtration through a Seitz filter (D-8 pad) and bottled in sterile containers. No merthiolate or other preservative was added.

Experimental Procedure

Twenty-one rabbits were injected with crystallized bovine serum albumin in the ear veins. The material was used in 25 per cent solution. Each dose consisted of 1 gm. (4 cc.) per Kg. of body weight. Twenty

animals survived for the duration of the experiments. Their general condition remained excellent, no loss of weight developed and no abnormalities of behavior were noted. One rabbit received seven injections in 14 days, during which it remained in good condition. On the second day after the seventh injection, the rabbit appeared sick and death occurred on the third day. Autopsy showed that death was due to bronchopneumonia. Since considerable postmortem change had taken place in the organs, the tissues from this animal were not used for detailed study.

The 20 rabbits in the series received crystallized bovine serum albumin (1 gm. per Kg. of body weight) as follows:

8 received a single injection and were sacrificed after 2, 6, 24, 48 (2 animals) and 72 hours, and 7 and 15 days;

4 received two injections 48 hours apart and were sacrificed 24, 48 and 72 hours, and 11 days after the second injection;

6 received seven injections in 15 days and were sacrificed 1, 4, 6, 9 and 18 (2 animals) days after the last injection;

2 received twelve injections in 25 days and were sacrificed 5 and 28 days after the last injection.*

The rabbits were killed with an overdose of chloroform and autopsied immediately. Rabbits kept under the same conditions, fed the same diet and sacrificed in the same way, but receiving no injections, were used as controls.

Thin sections of all organs were fixed as soon as possible in large quantities of Zenker's fluid and in 4 per cent solution of formaldehyde in physiologic saline. The tissues fixed in Zenker's fluid were embedded in paraffin and stained with eosin and methylene blue or phloxine and methylene blue. Sections of the spleen and kidney in several instances were also stained by the Turnbull's-blue method for iron. Portions of the spleens of two animals were fixed in corrosive sublimate and acetic acid for Feulgen's stain.[9] This technic, however, gave better results on tissues fixed in Zenker's fluid. For this reason, Zenker's-fixed tissues were used by preference for studies by Feulgen's method. Frozen sections cut from formaldehyde-fixed tissue were stained with scarlet red for the demonstration of fat.

Effect of Albumin Injections on Blood Count

A rabbit received one intravenous injection of bovine serum albumin; the red blood cell count, white blood cell count and differential count were made before injection and at half-hour intervals for 6½ hours. No significant variation was found in any of the counts.

* This would correspond to a total dosage of 840 gm. in a man weighing 70 Kg.

Two rabbits were given seven intravenous injections of bovine albumin in 15 days; blood counts were made after the second and each subsequent injection and at intervals of 4 days for 20 days after the last injection. No significant variation developed in the red blood cell count, white blood cell count or differential count.

Tissues Showing Little or No Alteration

Heart. The myocardial fibers were normal in all rabbits. In 4 of the rabbits (2 of those receiving one injection and 2 receiving seven injections) the number of lymphocytes and mononuclear cells in the myocardial connective tissue was slightly greater than that seen in the control rabbits. Since the same types of cells were found in varying numbers in the myocardial connective tissue of the controls, no particular significance was attached to this observation.

Lung. In 12 rabbits (4 with one injection; 2 with two injections; 6 with seven injections) there were small fresh hemorrhages without inflammatory reaction. Such small fresh hemorrhages are frequently seen in the lungs of rabbits sacrificed after many types of experimental procedures. There was no correlation between dosage of albumin and their occurrence, or between them and the interval separating the time of the last injection and the time of autopsy. No hemosiderin deposits or other evidences of old hemorrhage were found in any of the animals. For these reasons, we do not feel that the hemorrhages are related to the injections of bovine serum albumin. The trachea was normal in all rabbits in the series.

Gastrointestinal Tract. The esophagus, stomach, small intestine and colon were examined. These organs were normal in all rabbits.

Pancreas. Normal in all animals.

Adrenals. There were very small numbers of polymorphonuclear leukocytes scattered in the connective tissue of the adrenal cortex in 3 animals. No necrosis of the cortical parenchyma was found. The medulla was normal in all instances.

Ureter. Normal (examined in 2 animals).

Urinary Bladder. Normal in all animals.

Testis. Nineteen of the rabbits were males. The testes of 7 of these showed slight evidence of vitamin E deficiency. Similar changes were present in some of the control animals. These alterations are therefore regarded as the result of insufficient alpha-tocopherol in the diet rather than the consequence of the injections of bovine serum albumin. There were no testicular changes which could not be accounted for in this way.

Ovary and Uterus. These organs were normal in the 1 female rabbit in the series.

Thyroid. The thyroid was examined in 6 rabbits (1 which received one injection; 1, two injections; 3, seven injections; 1, twelve injections). It was normal in each instance.

Thymus. The thymus was negative in 6 rabbits (2 which received one injection; 1, two injections; 2, seven injections; 1, twelve injections).

Aorta. The aorta was normal in all rabbits of the series.

Abdominal Skin. Negative in all animals.

Musculature. The psoas muscle and muscles of the abdominal wall were examined in each rabbit. These were normal in all instances.

Central Nervous System. The cerebral cortex, hippocampus, brain stem, cerebellum and spinal cord were examined routinely. These levels of the central nervous system were normal in all the rabbits receiving one injection. All 4 rabbits to which two injections had been given showed a very small number of polymorphonuclear leukocytes in the central nervous system, unaccompanied by necrosis of cerebral tissue. In the rabbit autopsied 24 hours after the second injection, the cells were found at all levels examined. They were present in each of the sections except that of the spinal cord in the animal sacrificed 48 hours after the second injection. In the rabbit autopsied after 72 hours, there were increased numbers of polymorphonuclear leukocytes within the blood vessels of the cerebral cortex, hippocampus and brain stem, but none in the brain tissue itself. A very few cells of this type were found in the interstitial tissue of the cerebral cortex and hippocampus of the rabbit sacrificed 11 days after the second injection. There were no similar changes in any of the rabbits receiving seven injections or those with twelve injections. The rabbit receiving seven injections and autopsied 9 days after the last injection showed the lesions of spontaneous encephalitis of rabbits.

Tissues with Changes Associated with Increased Destruction of Blood Cells

Spleen. The spleen was slightly to moderately enlarged in each of the animals of the series autopsied within 10 days after cessation of the injections. In rabbits autopsied later the spleens showed no significant deviation from the normal on gross inspection.

The changes in the spleen have been interpreted as the result of an increase in phagocytosis of leukocytes and red blood cells in the splenic pulp. The differences between the spleens of the animals receiving injections of crystallized bovine serum albumin and those of normal controls are quantitative rather than qualitative.

In the spleen of the rabbit sacrificed 2 hours after a single injection of albumin, polymorphonuclear leukocytes in increased number were

found scattered through the pulp. Most of these cells were normal in morphology but many had nuclei which took the nuclear stain less deeply than usual. Occasionally, polymorphonuclear leukocytes were found in phagocytic cells. Both eosinophilic and neutrophilic varieties were affected. Fragments in the form of small, round, densely basophilic droplets were found in the pulp in moderate numbers. The droplets were, for the most part, in phagocytic cells, but some of them appeared free in the meshes of the pulp. Occasionally, the droplet-filled phagocytes were present lining the sinusoids and within their lumina. These droplets, whether intracellular or extracellular, were mostly grouped in foci; these were more abundant in the zone beneath the capsule than in the central portions of the pulp. By study of many microscopic fields, it was possible to find transitional stages between the droplets and the nuclei of degenerating polymorphonuclear leukocytes. The cytoplasm disappeared first and left no demonstrable trace. The granules of eosinophils often persisted longer than other cytoplasmic constituents. A few nuclei of polymorphous shape without cytoplasm were found in phagocytes.

Additional evidence that the basophilic droplets were of nuclear origin was afforded by study of sections stained by Feulgen's technic. Feulgen's method stains thymonucleic acids purple. Since thymonucleic acids occur in living tissues only in chromatin, the technic is specific for nuclear material. The droplets under consideration were stained purple by this method and the transitional stages were especially well shown. Similar droplets were also present in the spleens of normal control rabbits (Fig. 1). We have interpreted these findings as indicating that the rate of destruction of leukocytes, normally taking place in the rabbit spleen, was somewhat increased by the intravenous injection of crystallized bovine serum albumin and that this increase was already evident 2 hours after the time of injection.

The spleens of the rabbits sacrificed 6, 24 and 48 hours after a single injection showed progressive stages in the degeneration of polymorphonuclear leukocytes, apparently without further increase in the number affected. The partly degenerated, but partially intact, leukocytes were less and less frequent, deeply basophilic droplets being found in their stead. Seven days after injection there were only a few partially degenerated polymorphonuclear leukocytes, but the droplets were still present in considerable excess over the numbers in the normal spleen. Partial digestion of the droplets of nuclear material in the phagocytes was suggested by further fragmentation and decrease in size. In the rabbit sacrificed 15 days after a single injection, the quantities of degenerating polymorphonuclear leukocytes and of basophilic

droplets were essentially those of the spleens of normal control rabbits.

Phagocytosis of red blood cells was not increased to a significant degree in the spleens of rabbits sacrificed 2 and 6 hours after a single injection. This process, however, was conspicuous in the animals autopsied after 24 hours. Red blood cells, singly or in groups, were found in phagocytic cells of the pulp and in the sinusoids. Phagocytosis of red blood cells was present also in the spleens of animals sacrificed at 48 hours, 72 hours and 7 days.

Small amounts of hemosiderin were present in the spleen of the rabbit sacrificed 7 days after a single injection. This material was found in phagocytic cells, usually in the pulp but occasionally in the sinusoids. A few hemosiderin-filled phagocytes were seen in the spleens of normal control rabbits, but the increase in the experimental animal was definite. The amount of hemosiderin in the spleen of the rabbit autopsied 15 days after a single injection was less than it was in the animal sacrificed after 7 days but it was still considerably above the upper limit of normal. The hemosiderin appeared to be derived from degeneration of the phagocytosed red blood cells.

The same cells were concerned in the phagocytosis of red blood cells and of polymorphonuclear leukocytes. Some contained only red blood cells, others only degenerating leukocytes; at times, a single phagocyte contained both, or their degeneration products.

There was no definite change in the size, architecture, or cellular composition of the malpighian corpuscles of the several animals sacrificed after a single injection of albumin. The central arterioles, veins, trabeculae and capsule showed no essential variation from the normal. There was a variable degree of dilatation of the sinusoids. Except for the sequences described previously, the cellular composition of the pulp varied only within limits seen in normal control animals.

The spleens of the animals which received two, seven and twelve injections of crystallized bovine albumin showed changes differing only in degree from those already described (Figs. 2, 3 and 4). Separation of the several stages in the degeneration of polymorphonuclear leukocytes and red blood cells was rendered less clear in the rabbits receiving multiple injections of albumin. This was interpreted as an indication that each separate injection led to the degeneration of additional cells of both types. Thus, when a rabbit was sacrificed 24 hours after the last of seven injections, some of the stages of blood cell phagocytosis resembled those seen 24 hours after a single injection. Other stages corresponded to those found in rabbits autopsied at longer intervals after administration of albumin. In addition to the sequences connected with blood cell degeneration, there was a definite but moder-

ate hyperplasia of the malpighian corpuscles in the spleens of rabbits receiving seven and twelve injections of albumin (Fig. 5). These structures showed active proliferation of the cells in the germinal centers. In spite of the additive character of the changes resulting from several injections of albumin, the spleens again returned to normal after a few weeks. For instance, in the spleen of the rabbit sacrificed 28 days after the last of twelve injections, occasional hemosiderin-filled phagocytes were the only traces of blood cell phagocytosis.

In summary, the changes in the spleen are those of quantitative increase over the normal rate of blood cell destruction in this organ. Within a few weeks, the sequences progress to complete disappearance of the degeneration products of leukocytes and red blood cells. The changes in the spleen induced by injections of crystallized bovine albumin are, therefore, entirely reversible.

Mesenteric Lymph Nodes. The lymph nodes of rabbits, especially those of the mesentery, presented considerable variation in size, amount of lymphoid tissue and degree of sinusoidal dilatation under normal conditions. The mesenteric lymph nodes of the rabbits in the present series showed variations which were not beyond those of a similar group of control animals. These variations could not be correlated with the number of injections or the interval between the last injection and the time of autopsy.

Within and around the sinusoids, the same sequences were seen as in the spleen. There was phagocytosis of polymorphonuclear leukocytes with progressive disintegration, nuclear fragments persisting longer than the rest of the cell. Red blood cells were also phagocytosed and hemosiderin appeared as the result of their breakdown. The time relationships, including the time of return to normal, were the same in the lymph nodes as in the spleen. This process of phagocytosis was more extensive in the spleen than in the lymph nodes. In the few instances in which lymph nodes other than the mesenteric were examined, identical sequences were found.

Liver. There was coccidiosis in the livers of 6 rabbits. Otherwise, the general architecture of the liver was entirely normal. No changes suggesting storage of albumin were found.

Phagocytosis of polymorphonuclear leukocytes and red blood cells by Kupffer cells occurred to a limited extent. By careful search, evidence of such phagocytosis could be found in the Kupffer cells in normal control rabbits. Here, again, the differences between normal organs and those of the experimental animals were quantitative rather than qualitative. The sequences were identical with those described in the spleen. Phagocytosis of polymorphonuclear leukocytes was

noted earlier than that of red blood cells. In the animals allowed to survive longer after injection, nuclear fragments and hemosiderin made their appearance; these were not increased over the normal in animals sacrificed at the longest times after cessation of injections.

Bone Marrow. There was no definite change in the cellularity of the marrow in rabbits receiving one, two and seven injections.

A slight increase in cellularity of the marrow was present in the animal examined 5 days after the last of twelve injections (compare Figs. 6 and 7). The proportion of adult polymorphonuclear leukocytes was slightly increased in the marrow of rabbits receiving seven and twelve doses. In the rabbit sacrificed 28 days after the last of twelve injections, the marrow was normal in regard to number and character of cells.

Phagocytosis of adult blood cells occurred in the marrow of several rabbits to a very slight extent, the sequences being the same as those already described for the spleen, Kupffer cells and lymph nodes. Small amounts of nuclear débris and hemosiderin made their appearance as the result of blood cell degeneration, but these were not found in the animals allowed to survive longest after completion of the injections.

The Kidneys

The changes in the kidneys of rabbits injected with bovine serum albumin differ from those concerned with the degeneration and phagocytosis of polymorphonuclear leukocytes and red blood cells. In the kidneys of animals given a single injection of albumin and sacrificed at the various intervals listed under *Materials and Methods,* the glomeruli, blood vessels, collecting tubules, pelvic epithelium, renal capsule and stroma were entirely normal in appearance. There was slight swelling of the cells lining the ascending limbs of Henle's loops and the adjacent portions of the distal convoluted tubules, with dispersion of the cytoplasm. This swelling and concomitant dispersion of the granular cytoplasm was present to a degree only slightly beyond that seen in some areas of the kidneys of normal controls. It was evident in the kidneys of rabbits sacrificed 24, 48 and 72 hours after injection. The distribution of these minimal changes was fairly uniform; they were found to an equal degree in the central portions and at the poles. The lumina of the tubules contained very small amounts of granular precipitate similar in appearance and amount to that seen in controls. The other portions of the renal tubules in these rabbits were free from change. The kidneys of animals sacrificed at 2 hours, 6 hours and 15 days following a single injection showed no essential deviation from the normal.

In the kidneys of the rabbits sacrificed 24 hours after the second of two injections, there was a slight to moderate swelling of the tubular cells and a somewhat greater dispersion of the cytoplasm than was seen after one injection. These changes involved the ascending limbs of Henle's loops, the adjacent portions of the distal convoluted tubules and, in addition, portions of the proximal convoluted tubules.

Examination of the kidneys of the rabbit autopsied 48 hours after the second of two injections of albumin showed that there was further swelling of the same cells as were affected in the rabbit sacrificed after 24 hours. In several small areas, the swelling and dispersion of the cytoplasm resulted in the formation of large clear cells with a narrow rim of cytoplasm and the nucleus basally placed. The large central portion of these cells was entirely clear; the vacuoles did not contain fat in sections stained with scarlet red. A few fine droplets at the bases of the cells were colored red by this method.

The enlargement of these cells resulted in marked narrowing or even obliteration of the lumina of the tubules lined by them. The transition from slightly swollen tubular cells to the large clear cells was usually abrupt.

Evidence that the processes leading to the appearance of the large clear cells do not lead to necrosis of the cells was found in the absence of nuclear degeneration and of mitotic figures in the adjacent tubular epithelium. No leukocytic infiltration was seen in association with these cells. The absence of these cells with clear cytoplasm in animals allowed to survive longer after the last injection of albumin suggested that the process was reversible.

Changes similar in appearance, degree and frequency to those in the rabbit autopsied at 48 hours after the second injection were seen in the animal sacrificed after 72 hours. The appearance of the tubular epithelium in the rabbit autopsied 11 days following the second of two injections was essentially that of the control animals.

In all of the animals receiving two injections of crystallized albumin, the tubules contained very small amounts of granular precipitate, similar to that seen in control rabbits. The glomeruli, stroma, blood vessels, collecting tubules, pelvic epithelium and renal capsule were normal.

The kidneys of the animals sacrificed at various intervals after seven injections showed changes of essentially the same character as those described in the preceding series of rabbits, the differences being those of degree. Those seen in the animal autopsied 24 hours after the seventh injection were moderate to marked swelling of the cells lining nearly all ascending limbs of Henle's loops as well as those of the

distal convoluted tubules, descending limbs of Henle's loops and, to a less degree, the proximal convoluted tubules (Fig. 9). There were hyaline casts in the lumina of occasional tubules, for the most part in the descending limb of Henle's loop; there were a few in the collecting tubules. The amount of granular precipitate in the proximal and distal convoluted tubules was considerably increased over the amount seen in normal control rabbits. A small amount of granular precipitate was seen also in the lumina of some collecting tubules.

In the rabbit autopsied 4 days after the last of seven injections there were changes qualitatively similar to those just described, but markedly less in degree. Relatively few of the cells lining the tubules showed the clear cytoplasm with basally placed nucleus. The tubular cells were, on the average, smaller than equivalent cells in the animal sacrificed 1 day after the last of seven injections. The amount of granular precipitate in the convoluted tubules and in Henle's loops was less than in the rabbit autopsied at 24 hours, but was still in excess of that seen in control animals. No casts were present.

There were scars in the kidney of the rabbit sacrificed 9 days after the last of seven injections. These scars were not seen in any of the other animals of the series; they can be said with certainty to have antedated the experiments. Where uninvolved by the scarring, the renal tubules showed no significant differences from those of normal control animals.

The kidneys of both rabbits sacrificed 18 days after the last of seven injections showed a few groups of swollen cells with clear cytoplasm and basally placed nuclei in the distal convoluted tubules and ascending limbs of Henle's loops. The remainder of the tubules were normal in appearance. The glomeruli, blood vessels, stroma, collecting tubules, pelvic epithelium and renal capsule were normal in the animals receiving seven injections, except for the already described areas of scarring in 1 rabbit.

One of the 2 animals receiving twelve injections was sacrificed 5 days after the last injection. There were infrequent segments of the distal convoluted tubules or adjacent limbs of Henle's loops in which clear swollen cells were present. These changes were identical with those previously described; the general appearance of the kidney was similar to that of the kidney in the rabbit sacrificed 6 days after the last of seven injections. In the kidney of the animal autopsied 28 days after the last of twelve injections, there were very rare groups of clear cells in the tubules (not more than three in a section including the entire kidney). Except for these groups of cells, the kidneys were entirely normal (Fig. 10).

Discussion

This study has been designed primarily to determine whether crystallized bovine serum albumin injected intravenously in rabbits is stored in tissues for considerable periods of time, whether there are morphologic changes in the tissues of the experimental animals which can be attributed to the injections of albumin and whether the consequences of repeated injections are different from those of one injection. In addition, in this study we have attempted to determine whether crystallized albumin may be used for investigation of the biology of tissues.

Crystallized bovine serum albumin is not stored for any considerable period of time in the tissues of the rabbit in such form as to be recognized by morphologic methods. In this, albumin differs from solutions of many other compounds of high molecular weight, such as gum acacia and polyvinyl alcohol.[10] Since the bovine albumin is not excreted in the urine but disappears slowly from the circulating blood without being stored as such in the tissues, it is presumed to be used up in metabolism or to be incorporated, either with or without change, in the structure of protoplasm. The technics used in this study are not sufficient to distinguish between these possibilities.

The general condition of the animals remained excellent throughout the experiments. Weight was maintained and no abnormalities of behavior developed. Repeated injections, up to twelve, had no more effect on the general condition of the rabbits than did a single injection. The relation of multiple injections to tissue reactions is discussed elsewhere. It should be emphasized that the amounts of albumin per kilogram of body weight were large—up to 12 gm. per Kg. This is in excess of the amount likely to be required in the treatment of human patients and is also greater than the quantity necessary to produce striking tissue changes with gum acacia, polyvinyl alcohol and other large-molecular compounds.[10]

The morphologic changes in the tissues of rabbits receiving intravenous injections of bovine serum albumin fall into two groups. The first group consists of changes in the spleen and other organs of the reticulo-endothelial system; the second group is concerned with alterations in the kidneys.

The histologic changes in the spleen and to a less degree in the liver, lymph nodes and bone marrow depended upon tissue sequences concerned with the degeneration and phagocytosis of red blood cells and polymorphonuclear leukocytes. This process was seen in a small number of cells in the same organs of normal rabbits and the sequences are identical in the control and in the experimental animals. For this

reason, the alterations are interpreted as the result of a quantitative increase in the rate of a normal process.

MacKenzie, Whipple and Wintersteiner [11] have shown that the interstices of the splenic pulp act as a filter separating blood cells from the plasma. Some cells of the blood pass rapidly through the meshes of the pulp, while others remain there for considerable periods of time, as in eddies alongside a swiftly moving current. For this reason, part of the blood cells are exposed to the phagocytes of the splenic pulp much longer than are others. The phagocytes under normal conditions take up a few red blood cells and leukocytes. These cells disintegrate in the phagocytes. Hemosiderin is produced from the degenerated red blood cells (Fig. 2) and nuclear fragments persist after the other portions of leukocytes can no longer be identified morphologically (Figs. 3 and 4).

The intravenous injection of crystallized bovine serum albumin in rabbits leads to increased destruction of blood cells in the spleen. This increase is noted first in the case of the polymorphonuclear leukocytes, being evident in the animal sacrificed 2 hours after a single injection. Phagocytosis of red blood cells was not definitely increased over the normal in rabbits autopsied 2 and 6 hours after a single injection but was demonstrated 24 hours after injection. We have not been able to determine as yet the actual mechanism by which intravenous injection of heterologous albumin leads to an increase in the amount of normal blood cell phagocytosis in the spleen. Studies on normal human subjects indicate that there is a very rapid adjustment to the physiologic changes brought about by the intravenous injection of crystallized bovine albumin. These studies have not been repeated on the rabbits in this series. However, it seems logical to assume that the increase in osmotic pressure caused by the albumin is compensated within a few minutes in the rabbit, as in man. Since histologic evidence of red blood cell destruction in abnormal amounts is not found for several hours after administration of albumin, it seems unlikely that the changes in osmotic pressure or in blood volume are directly related to the increase in rate of phagocytosis of red blood cells. A more probable explanation, but one not established by this study, is that the intravenous injection of bovine albumin in rabbits produces flocculation of relatively small numbers of red blood cells which are then separated from the plasma in the splenic pulp and broken down over a period of time.

Since increased phagocytosis of leukocytes appears at a significantly earlier time after injection of bovine albumin than does phagocytosis of red blood cells, the degeneration of leukocytes may depend either

upon a different mechanism from that affecting the red blood cells or the leukocytes may succumb more quickly to a similar alteration. The leukocytes of the rabbit are known to be affected more easily by a number of pathogenic agents than are the leukocytes of several other species of animals commonly used in the laboratory.

Sequences of phagocytosis of red blood cells and leukocytes were also found to a much smaller extent in the mesenteric lymph nodes, bone marrow and Kupffer cells of the liver. They would appear, therefore, to be concerned with the reticulo-endothelial system or a considerable number of its components. The increase in rate of blood cell phagocytosis, while definite, does not affect a large proportion of the blood cells, as shown by the fact that counts on the circulating blood do not change significantly. There is only a slight hyperplasia of the bone marrow, even after twelve injections.

When a series of injections of bovine albumin are given intravenously, each injection initiates a new set of the same sequences. The tissues of rabbits autopsied after several injections show some sequences near the final stage of disappearance of hemosiderin and nuclear particles and others at the stage of fresh phagocytosis of blood cells. With the cessation of injections, the sequences go on to conclusion and the organ returns to the state in which it was before albumin has been administered. The changes induced in the spleen and other organs of the reticulo-endothelial system are entirely reversible, so far as each organ as a whole is concerned. The changes in the individual phagocytosed cells are, of course, irreversible.

The histologic sequences in the kidneys of rabbits receiving bovine albumin are different from the sequences found in the spleen and other organs of the reticulo-endothelial system. The study of the morphologic changes in the kidney is best attacked by considering some of the facts of renal physiology. It has been demonstrated [12-14] that there is a renal threshold for such relatively large molecules as hemoglobin (molecular weight, 68,000). A state of equilibrium exists between the amount of hemoglobin absorbed by the tubular epithelium and the amount of hemoglobinogenous products removed from these cells per unit of time.[12] Direct measurements of the nature and amount of the protein in glomerular filtrates have been made.[15, 16] It is important to bear in mind that the presence of small quantities of protein in the glomerular filtrate, as noted in the literature, actually indicates that relatively large quantities of protein are filtered through the glomerulus and reabsorbed by the tubules when the amount of glomerular filtrate is compared with the amount of urine excreted.

These facts aid in understanding the morphologic changes in the

renal tubular epithelium as the result of intravenous injection of crystallized albumin. The studies on hemoglobin absorption are especially helpful since the molecular weight of hemoglobin is of the same order of magnitude as that of bovine albumin. The alterations noted histologically in the rabbits receiving bovine albumin involve the swelling and dispersion of the cytoplasm of the epithelium of the ascending limb of Henle's loop and the adjacent distal convoluted tubules. The most marked degree is found in animals sacrificed soon after several injections; in these animals the cytoplasm appears clear in tissue sections and the nucleus is displaced toward the basement membrane (Fig. 9). After a single injection, the changes are maximal between 24 and 72 hours; thereafter, they are regressive. Crystallized bovine serum albumin does not appear in the urine when injected intravenously in human patients and in dogs; we have not tested the urine of the rabbits used in this study.

The swelling and dispersion of the cytoplasm seem to be related definitely to the injections of bovine albumin. They are not found in the normal rabbit kidney; their degree can be directly correlated with the number of injections and the interval after the last injection. The changes appear to represent absorption rather than excretion. Confirmation of this view is found in the kidney showing maximal changes in tubular epithelium, and hyaline casts in addition. These casts were, for the most part, in the descending limb of Henle's loop and not in the ascending limb, convoluted tubules, or collecting tubules. It might be expected that casts would appear in, and distal to, the ascending limb of Henle's loop if the lining cells were excreting protein, rather than absorbing it. We feel, then, that some of the crystallized bovine albumin passes the glomerular filter in the rabbit's kidney at a rate comparable to that observed for such proteins as hemoglobin; it is then absorbed by the tubular epithelium and returned to the circulation, with or without chemical modification.

Particular attention has been paid to the condition of the aorta and large arteries of the organs in all the animals in view of Hueper's [10] studies on the relation of certain compounds of high molecular weight to vascular lesions. Hueper [17] administered polyvinyl alcohol intravenously and intraperitoneally in 5 per cent solution to dogs and found that atheromatous lesions in the aorta, carotid and femoral arteries resulted. This material was also found within the media of smaller arteries and arterioles of the heart and kidney. He regarded the lesions as morphologically similar to the cholesterol atheromatosis observed in man and rabbits. From these and additional experiments [18, 19] he concluded that the presence within the blood and tissues of abnormal

amounts of chemically inert macromolecular substances, which resist metabolic degradation and are therefore not readily eliminated through the filtration membranes, leads to the development of organic and particularly vascular lesions which display a certain degree of similarity in location and character.[10]

Study of the animals in this series indicates that such changes do not result from the intravenous administration of crystallized bovine albumin. The experimental animals in Hueper's experiments received amounts of polyvinyl alcohol per kilogram of body weight which are of the same order of magnitude as the amounts of crystallized albumin used in our experiments. Comparable time intervals were used in both sets of experiments. The actual excretion of crystallized albumin or of the products of breakdown of this substance has not been demonstrable. Since the albumin has been found to disappear from the blood stream slowly over the course of several days, it must be considered to be utilized in metabolism or stored. The absence of atherosclerosis in all of our experimental animals and, indeed, the absence of histologic evidence of deposition or storage of the albumin or of the products of disintegration of this substance are evidence that the prolonged presence of this exogenous macromolecular substance does not give rise to any of the changes which might be expected from it if all macromolecular substances were to give rise to the morphologic changes due to methyl cellulose and polyvinyl alcohol. The experimental evidence indicates that crystallized bovine serum albumin, although an exogenous material in the rabbit, is dealt with by the tissues in far different fashion from the macromolecular compounds studied by Hueper.[10] The size of the molecule is a secondary factor in determining the character, distribution and extent of tissue reaction to exogenous materials.

In view of Letterer's [20, 21] theory that amyloid is produced by a local reaction of antigen and antibody, we have searched for this material in the rabbits injected with crystallized bovine serum albumin. It has not appeared in any instance. The intervals during which the animals have been observed are not so long as would be useful in excluding completely the possibility of the formation of amyloid. Studies have been undertaken, to be reported in detail later, on the effect of very large amounts of crystallized bovine serum albumin in mice. The mouse is an experimental animal in which amyloid is produced quickly by a variety of agents, yet preliminary studies indicate that amyloid is not produced under these experimental conditions.

When the present study was begun, amorphous albumin containing a low percentage of other blood proteins was the purest preparation

available. Within a short time, crystallized albumin was prepared; the experiments were repeated with this material. Comparison of the tissues of animals injected with the two types of preparation indicates that the histologic changes resulting from amorphous bovine albumin were greater and, to a certain extent, different from those elicited by the crystallized material.

The availability of crystallized albumin furnishes a new biologic technic for study of many problems in tissue behavior. This study, undertaken from another point of view, has suggested that it may be used in investigation of problems in connection with the physiology and pathology of the spleen and kidney. Its value is enhanced by the fact that other blood proteins in highly purified form may be used in conjunction with the albumin or in comparison with it.

It is evident from these experiments that large and repeated intravenous injections of crystallized bovine serum albumin produce relatively minor histologic changes in the rabbit; the changes which are produced are reversible. In some of the animals, the total amount of albumin injected was approximately twice that of the total protein of the circulating blood of the rabbit. The use of crystallized bovine serum albumin in the rabbit involves a species difference between the animal from which the material was derived and that in which it was injected. Another species difference is involved in the use of such crystallized albumin in man; it is an assumption to apply these results directly to human patients. In so far as the experiments go, however, none of the histologic changes which resulted from the intravenous administration of crystallized bovine albumin can be regarded as a contraindication for the use of the material.

Summary

Rabbits were injected intravenously with crystallized bovine albumin in 25 per cent solution. The dosage was 1 gm. of albumin per Kg. of body weight. Animals were sacrificed at various intervals after one, two, seven and twelve injections.

The injections of crystallized bovine albumin induced no change in state of nutrition or behavior of the rabbits.

The changes in tissues attributed to the albumin were confined to the kidneys, bone marrow, spleen and, to a less degree, to other organs of the reticulo-endothelial system.

Slight enlargement of the spleen was noted grossly. Histologically, there was an increase in the amount of phagocytosis of leukocytes and of red blood cells in the pulp and sinusoids of the spleen. This was

regarded as an accentuation of the process occurring in the spleen of normal rabbits.

Similar sequences of phagocytosis of blood cells were found in the Kupffer cells of the liver, in the lymph nodes and in the bone marrow. In these organs, the process took place to a very slight extent.

In the kidneys there was swelling and dispersion of the cytoplasm of the epithelium in the ascending limb of Henle's loop and in the distal convoluted tubules. These changes were regarded as an indication that some of the albumin was filtered through the glomerulus and entered the tubular epithelial cells more rapidly than it was reabsorbed in the circulating blood.

When injections were repeated, each injection gave rise to another series of tissue sequences which were identical with those resulting from one injection.

The histologic changes were reversible, the organs returning to a normal state even after twelve injections.

Amyloid was not found in any of the rabbits.

Crystallized bovine serum albumin gives rise to somewhat less marked tissue reaction in rabbits than does purified amorphous bovine albumin.

Crystallized bovine serum albumin is not stored in the tissues of rabbits in a form recognizable morphologically. In this respect it differs from methyl cellulose, polyvinyl alcohol and certain other compounds of high molecular weight.

Because of species differences, it is hazardous to assume that the tissue reactions of man to crystallized bovine serum albumin are the same as those of the rabbit. However, no tissue reactions have been found in the rabbit which would contraindicate the use of crystallized bovine serum albumin, were they to occur in human patients.

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[*Illustrations follow*]

DESCRIPTION OF PLATES

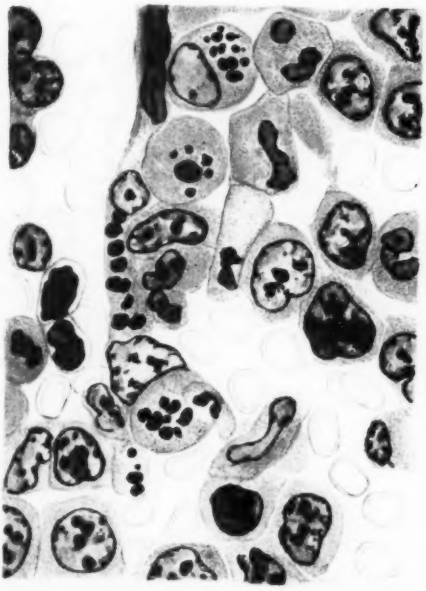
PLATE 33

FIG. 1. Spleen of control rabbit. Lining a sinusoid and lying free in the pulp there are phagocytes containing fragments of nuclear material. Several erythrocytes are seen in the open meshwork of the pulp. Camera lucida drawing. Phloxine and methylene blue stain. × 945.

FIG. 2. Spleen of rabbit sacrificed 1 day after the last of seven injections. Several phagocytes contain varying amounts of hemosiderin. In addition, a few red blood cells are seen phagocytosed whole, not yet having been degraded to hemosiderin. Camera lucida drawing. Turnbull's-blue stain for hemosiderin with basic fuchsin counterstain. × 660.

FIG. 3. Spleen of rabbit sacrificed 4 days after the last of twelve injections. Within the pulp are two large phagocytes laden with fragments of nuclear material. In one of these a phagocytosed polymorphonuclear leukocyte is seen to be undergoing degenerative changes. Camera lucida drawing. Phloxine and methylene blue stain. × 945.

FIG. 4. Spleen of same rabbit as used for Figure 3. The identity of the nuclear fragments is established by the purple coloration with Feulgen's stain. There are two intact polymorphonuclear leukocytes apparently beginning to undergo degeneration. Camera lucida drawing. Feulgen's stain, Licht Grün counterstain. × 945.



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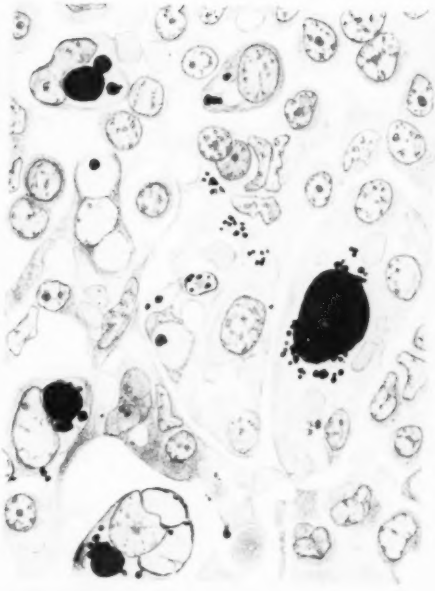

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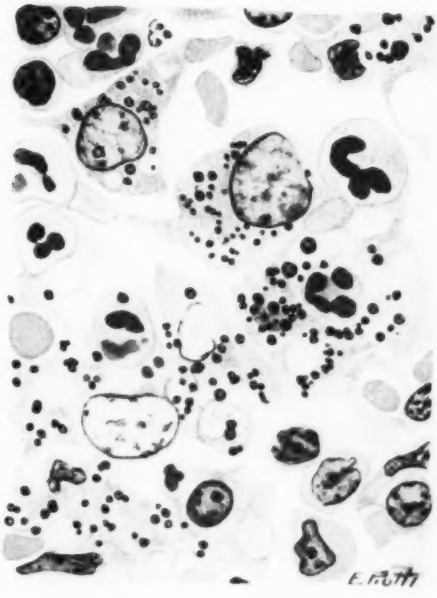

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Bailey and Hawn

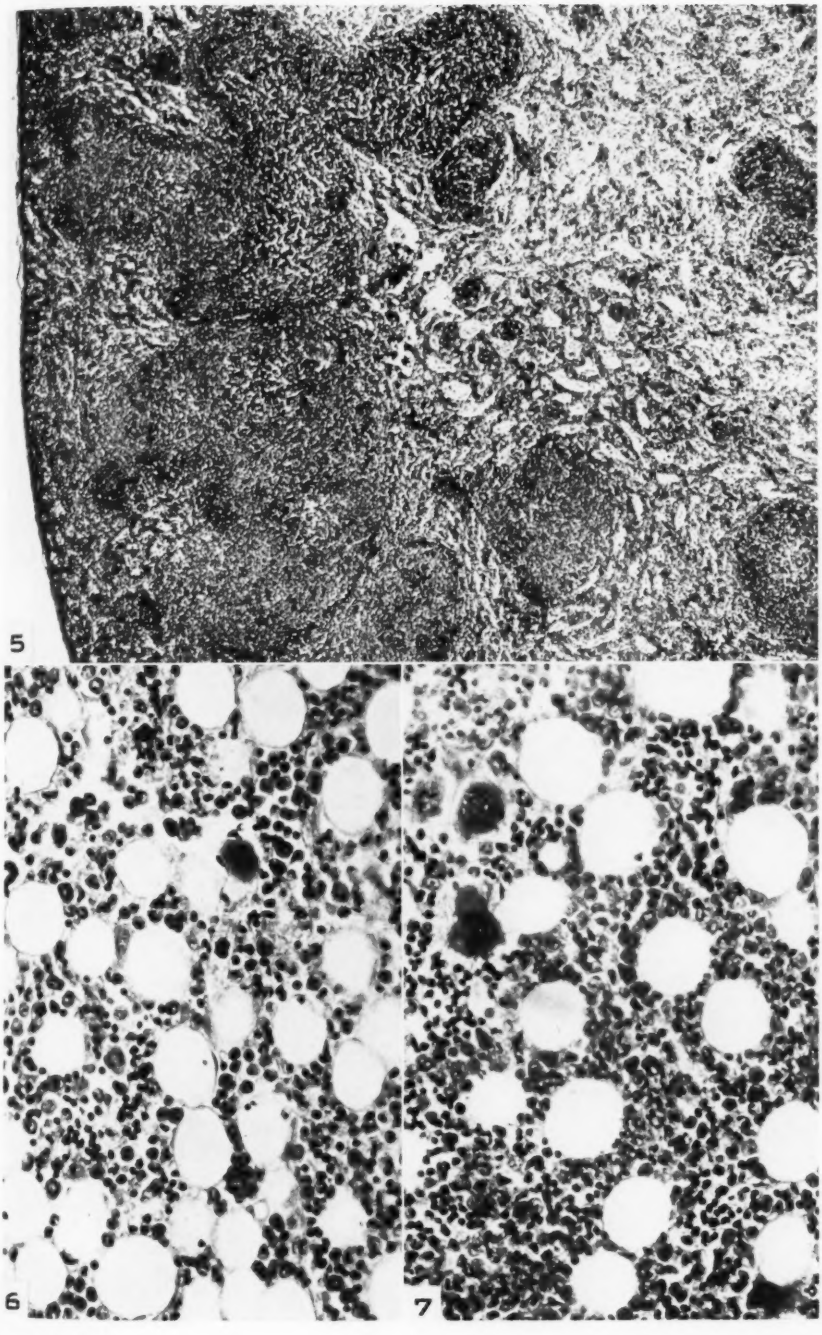
Effect of Crystallized Bovine Serum Albumin

PLATE 34

FIG. 5. Spleen of rabbit sacrificed 5 days after the last of twelve injections. The sequences of degeneration and phagocytosis of erythrocytes and leukocytes have caused no significant alteration in the general architecture. The malpighian corpuscles are moderately prominent and the pulp is somewhat increased in cellularity, particularly beneath the capsule. Phloxine and methylene blue stain. × 45.

FIG. 6. Femoral bone marrow of control rabbit. Phloxine and methylene blue stain. × 300.

FIG. 7. Femoral bone marrow of animal autopsied 5 days following the last of twelve injections. There is slight to moderate increase in the cellularity, with slight increase in the proportion of adult polymorphonuclear leukocytes. General architecture remains normal. Megakaryocytes show no unusual features. This is the most marked degree of hyperplasia in any animal of the series. Phloxine and methylene blue stain. × 300.



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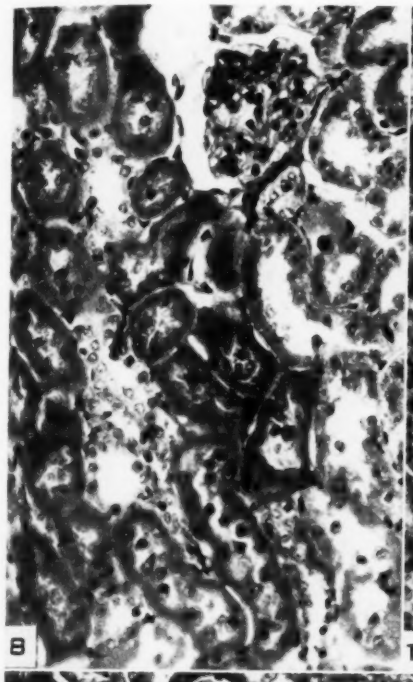
Bailey and Hawn Effect of Crystallized Bovine Serum Albumin

PLATE 35

FIG. 8. Kidney of control rabbit. Phloxine and methylene blue stain. × 300.

FIG. 9. Kidney of rabbit sacrificed 24 hours after the last of seven injections. The distal convoluted tubules, the ascending limbs of Henle's loops and portions of the proximal convoluted tubules show swelling and vacuolation of the lining cells with encroachment upon or obliteration of the lumina. There are no evidences of cell necrosis. Granular protein precipitate is seen in tubular lumina. The glomerulus shows no abnormalities. Phloxine and methylene blue stain. × 300.

FIG. 10. Kidney of rabbit sacrificed 28 days after the last of twelve injections. The tubular epithelium is essentially normal. No scarring, cellular infiltration, or reparative proliferation is evident. The glomerulus is normal in appearance. Phloxine and methylene blue stain. × 300.



Bailey and Hawn Effect of Crystallized Bovine Serum Albumin

THE EFFECT OF POSTURAL HYPERTENSION ON THE DEVELOPMENT OF ATHEROMATOSIS IN RABBITS FED CHOLESTEROL *

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In the course of some experiments originally designed to determine the effects of posture on the distribution of atheromatous lesions in the arteries of rabbits on high cholesterol diets, it was observed that rabbits maintained in an upright position for several hours daily during the feeding period developed more abundant lesions than control animals. There are a number of ways in which the change in posture may produce this result. The metabolism of cholesterol may be changed, the arterial wall may be subject to greater stress and strain and, finally, altered dynamics of blood flow may be implicated. In this report data are presented which indicate that elevation of blood pressure is the chief mechanism involved.

MATERIAL AND METHODS

Six adult rabbits, weighing 2500 gm. each, were placed in upright sitting position for 5-hour periods, 6 days per week. During this time they received 1 gm. of cholesterol 6 days per week. The cholesterol was dissolved in 6 cc. of hot olive oil and made into a paste with bran according to the method of Harrison.[1] This diet was supplemented with commercial rabbit chow and fresh vegetables. Two rabbits were sacrificed after 4 weeks, two after 8 weeks and two after 12 weeks.

Two adult rabbits, weighing 2500 gm. each, were maintained in upright sitting position for 5-hour periods 6 days a week for 12 weeks, but were not fed cholesterol.

Seven adult rabbits, weighing 2500 gm. each, were fed 6 days a week 1 gm. of cholesterol dissolved in 6 cc. of hot olive oil absorbed in bran flakes. These were kept under usual laboratory conditions in individual standard rabbit cages. Two were sacrificed after 8 weeks, four after 12 weeks and one after 17 weeks.

The rabbits were maintained in the upright position by placing them in cylindrical glass jars 32 cm. high and with an internal diameter of 14 cm. The bottoms of the jars were covered with a layer of sawdust about 3 cm. deep. The animals fitted into these jars rather snugly but without discomfort, their haunches resting on the sawdust layer and their heads protruding above the tops of the jars. They seldom struggled to escape and usually rested quite peacefully during the

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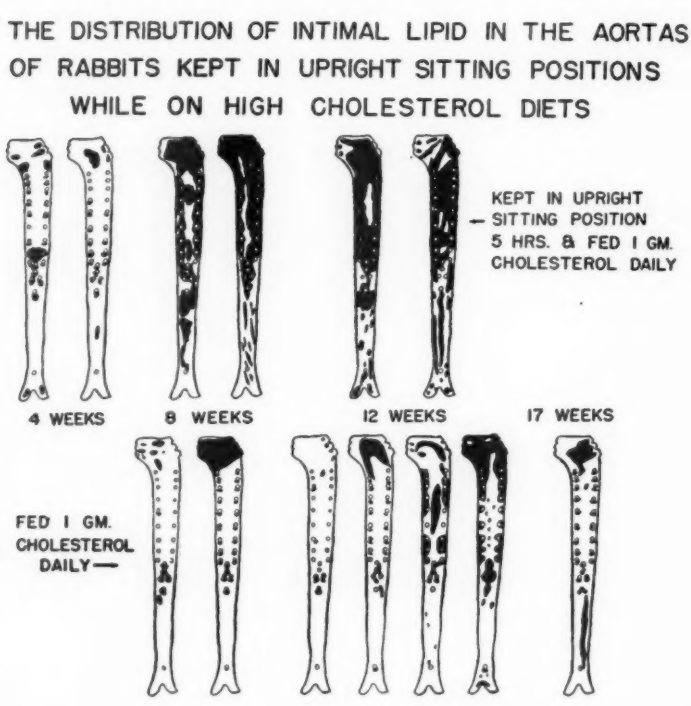
whole procedure. Occasionally after several weeks the rabbits learned to escape from the jars. Coarse chicken wire baskets, cylindrical in shape and 14 cm. high, were then fitted over the tops of the jars. The procedure seemed to have no deleterious effect on the general health of the animals. They ate well and actually gained weight.

At the time of sacrifice the aorta was stained *in toto* with Sudan IV and the size and distribution of intimal plaques mapped out diagram-


THE DISTRIBUTION OF INTIMAL LIPID IN THE AORTAS OF RABBITS KEPT IN UPRIGHT SITTING POSITIONS WHILE ON HIGH CHOLESTEROL DIETS
KEPT IN UPRIGHT SITTING POSITION 5 HRS. & FED 1 GM. CHOLESTEROL DAILY
4 WEEKS
8 WEEKS
12 WEEKS
17 WEEKS
FED 1 GM. CHOLESTEROL DAILY

Text-Fig. 1. The distribution of intimal lipids in the aortas of rabbits kept in upright sitting position while on high cholesterol diets. The shaded areas indicate lipid deposits. The aortas in the lower row are from rabbits fed with cholesterol but not subjected to postural change.

matically (Text-Fig. 1). The aortas were then fixed in Kaiserling I solution and photographed (Fig. 1). Subsequently they were washed in running water for 72 hours and placed in Zenker's solution. The entire vessel was rolled into a coil, embedded in paraffin, and representative sections were stained with hematoxylin and eosin, hematoxylin and Weigert's elastic tissue stain, Weigert's elastic tissue stain and van Gieson's stain, and by the Foot-Bielchowsky methods.

Results

The two animals sacrificed after 4 weeks of cholesterol feeding and daily periods in the vertical position showed macroscopic evidence of lipid deposition in the aorta. Previous experience with this feeding method has shown that after 4 weeks there are seldom any grossly visible plaques, although lipid-containing cells in small aggregates are usually demonstrable in the intima microscopically. The amount present in these two aortas was therefore abnormally great. The position of the lipid masses was not strikingly unusual, most of it being at the margins of branch orifices. Rather more lipid was noted in the lower thoracic portion and about the orifices of large abdominal branches and rather less in the ascending aorta than is usually the case.

After 8 weeks the four control animals showed intimal plaques of varying size and number. The largest ones were found in the ascending part of the aorta and the arch. In every instance large areas of the intima were free of lipid deposits. Both rabbits which were maintained in the upright position during 8 weeks of cholesterol feeding showed intimal plaques which were continuous or confluent over almost the entire aorta, so that the small lipid-free zones were entirely enclosed by lipid-containing areas which isolated them from each other. The plaques were abundant in the abdominal portion of the vessel although somewhat less so than in the proximal regions. The impression was thus gained that not only were lipid deposits greatly increased by the change in posture but that there was a tendency for them to appear in unusual locations. However, their presence in the abdominal aorta might be considered to be simply an expression of the greater total amount of fat deposited rather than a real reversal of the usual pattern observed in control animals.

After 12 weeks there was still a striking difference between the two animals which were fed cholesterol and maintained in the upright position and the two animals which were fed cholesterol but allowed normal posture. One animal fed cholesterol continuously for 17 weeks without being placed upright showed less atheroma than either of the two rabbits kept upright daily for 8 weeks.

In summary, rabbits maintained in upright sitting position in glass jars for 5 hours daily during cholesterol feeding developed larger intimal lesions than control animals fed with equivalent amounts of cholesterol. Furthermore, the plaques developed more quickly and tended to be more widely distributed in the aorta in the animals which were kept upright.

Three possibilities suggest themselves to account for these findings.

(1) Rabbits kept in the upright sitting position may develop hypercholesterolemia more rapidly than the controls. (2) The mechanical effects of abnormal posture may injure the aortic wall in such a fashion as to make it more vulnerable to lipid deposit. (3) The blood pressure may be increased during the period of postural change and the observed acceleration of lipid deposit may be due to relative hypertension. Each of these possibilities was investigated.

Cholesterol Content of Blood in Rabbits Maintained in Upright Sitting Position

Man and Peters [2] have shown that when the human subject is kept in the erect position and at rest for periods of 30 minutes there is a relative increase of plasma protein and cholesterol of over 10 per cent. This they attributed to an increase in tissue fluid at the expense of blood volume, the larger relatively impermeable molecules such as protein and cholesterol failing to pass through the capillary membranes and being retained in the circulation. Such a phenomenon, if present in rabbits kept in upright sitting position, might account for transient periods of elevated blood cholesterol and thus explain the increased tendency for atheromatous lesions to develop. Five rabbits were tested. Five cc. of blood were removed from ear veins just before the rabbits were placed upright in jars and again at the end of 5 hours. The blood obtained was analyzed for protein content by the specific gravity drop method of Drew, Scudder and Papps [3] and for cholesterol by the Schoenheimer-Sperry technic.[4] Four of the five rabbits were then fed 1 gm. of cholesterol in 6 cc. of olive oil daily. The test was repeated after 4 and 8 weeks. To control the factor of dilution of blood by bleeding, at the final test the first blood samples were taken at the termination of the upright sitting period and the second samples 15 hours later. The results are listed in Table I. They failed to show any consistent change in either protein or cholesterol levels which can be attributed to postural change. The level of blood cholesterol attained in the four rabbits which were fed with this material showed such variation both after 4 and 8 weeks that it is not possible to say whether these values are greater than those which might be observed in cholesterol-fed rabbits not subjected to changes in posture. Weinhouse and Hirsch [5] reported serum cholesterol values, not unlike those recorded here, in their rabbits which were fed approximately the same amounts of cholesterol. The amount of cholesterol deposited in extravascular sites did not differ appreciably between the rabbits kept in erect position and the controls. In fact the amount of lipid found in the aortic intima and other sites did not correspond closely to the

blood cholesterol content. It may be concluded that the observed increase in aortic lipid deposit in rabbits maintained in erect sitting position during cholesterol feeding periods is not dependent on a tendency toward hypercholesterolemia.

TABLE I

Serum Cholesterol and Protein in Rabbits Before and After Being Placed in Upright Sitting Position for 5 Hours

Rabbit no.	Position	Period of cholesterol feeding: None — Total cholesterol (mg. per 100 cc.)	None — Protein (gm. per 100 cc.)	4 weeks — Total cholesterol (mg. per 100 cc.)	4 weeks — Protein (gm. per 100 cc.)	8 weeks — Total cholesterol (mg. per 100 cc.)	8 weeks — Protein (gm. per 100 cc.)
1	Prone	66	6.83	290	5.81	...	5.37
	Upright	65	6.02	280	5.99	600	5.17
2	Prone	59	5.17	801	5.24	1430	5.27
	Upright	60	6.21	782	5.62	1430	4.99
3	Prone	61	5.69	460	5.27	625	5.85
	Upright	59	4.86	482	5.89	603	5.78
4	Prone	67	7.07	395	5.95	520	6.29
	Upright	64	6.05	385	5.48	530	5.58
5 Not fed cholesterol	Prone	64	6.46	63	6.43	58	6.50
	Upright	63	6.86	59	5.78	59	5.92

The Mechanical Effect on the Aorta of Maintaining Rabbits in Upright Sitting Position

Klotz [6] demonstrated that by suspending rabbits by their hind legs, he could accelerate the development of sclerotic changes in the aorta. The lesions produced were not unlike those produced by adrenalin injections and sometimes observed spontaneously. It has been shown (Anitschkow [7] and Harrison [1]) that the presence of medial defects in the rabbit's aorta will influence the deposition of intimal lipid following cholesterol feeding. In fact, Anitschkow [8] combined cholesterol feeding with suspension of the rabbits by their hind quarters but failed to observe any marked acceleration of lipid deposition.

The procedure used in these experiments is much less drastic than that employed by Klotz.[6] Nevertheless the aortas were studied histologically for evidence of structural change which might be attributed to the postural treatment alone. The aortas of two control rabbits which were kept erect but were not fed with cholesterol were available in addition to those of the other animals. Areas of medial fibrosis or scarring were occasionally observed but these were no more extensive

than in the control animals not subjected to postural change. There is, therefore, no morphologic evidence that mechanical trauma to the aorta is a factor in the increased deposition of lipid.

The Effect on the Blood Pressure of Maintaining Rabbits in Upright Sitting Position

Intra-arterial pressure is generally believed to be a factor in the development of human atheromatous lesions. Such lesions are found in parts of the vascular system where the pressure is normally relatively high and seldom in very small systemic arteries, pulmonary arteries, or veins. There is frequent association of hypertension and advanced arteriosclerosis. When conditions in the pulmonary circulation lead to increased intra-arterial pressure, intimal lipid-containing plaques frequently develop in this unusual site. In fact, some authors assign a primary rôle to the influence of hypertension on the development of arteriosclerosis. However, many subjects exhibit advanced lesions without clinical evidence of hypertension or anatomic evidence of cardiac hypertrophy. Another more striking discrepancy is the fact that individuals with "malignant" hypertension with the pressure markedly elevated for many years may die in renal insufficiency and, although the arteriolar bed may show striking changes, the larger arteries frequently present surprisingly few intimal lesions. Thus there is evidence to indicate that the presence of hypertension is a factor favoring the development of arteriosclerosis, but there is also good reason to believe that other conditions must likewise be fulfilled.

The rôle of the blood pressure in the development of the experimental lesions in cholesterol-fed rabbits is less clear. Anitschkow [8] claimed to have accelerated the development of these lesions by producing hypertension experimentally. He placed constricting ligatures on the abdominal aorta just above the bifurcation before cholesterol feeding was begun. However, it is not likely, according to the more recent findings of Goldblatt, Kahn and Hanzal,[9] that a ligature placed at a point so far beyond the renal orifices will result in sustained hypertension. It has been claimed that the feeding of cholesterol alone produces an elevation of blood pressure.[10] However, there are no convincing blood pressure measurements to prove this. Katz, Sanders, Megibow and Carlen [11] found normal blood pressures in rabbits on cholesterol diets even though the heart weights were increased at the time the animals were sacrificed. In a recent publication, Dill and Isenhour [12] reported that 7 of 12 rabbits with experimental renal hypertension of long duration developed aortic atheromatous plaques.

These rabbits were fed stock diets and failed to show elevation of blood cholesterol.

The pulmonary arteries in cholesterol-fed rabbits are almost as commonly the seat of lipid deposits as are the systemic arteries. This suggests that the blood pressure is of less significance in the pathogenesis of rabbit lesions than it is in human lesions.

Nevertheless, in order to determine if this might be a factor in the accelerated production of intimal lesions the blood pressure of ten normal and of five already hypertensive rabbits was measured indirectly by the ear capsule method of Grant and Rothschild [13] in the prone and subsequently in the upright sitting position. This method has been shown to give consistent results although the values obtained from the medial artery of the ear are considerably lower than those by direct cannulation of the carotid artery. The hypertensive rabbits had had one kidney wrapped in cellophane and the opposite one subsequently removed. The procedure has been shown by Page [14] and by Graef and Page [15] to be an effective method of producing hypertension in dogs.

TABLE II

The Effect of Posture on the Systolic Blood Pressure in Normal Rabbits

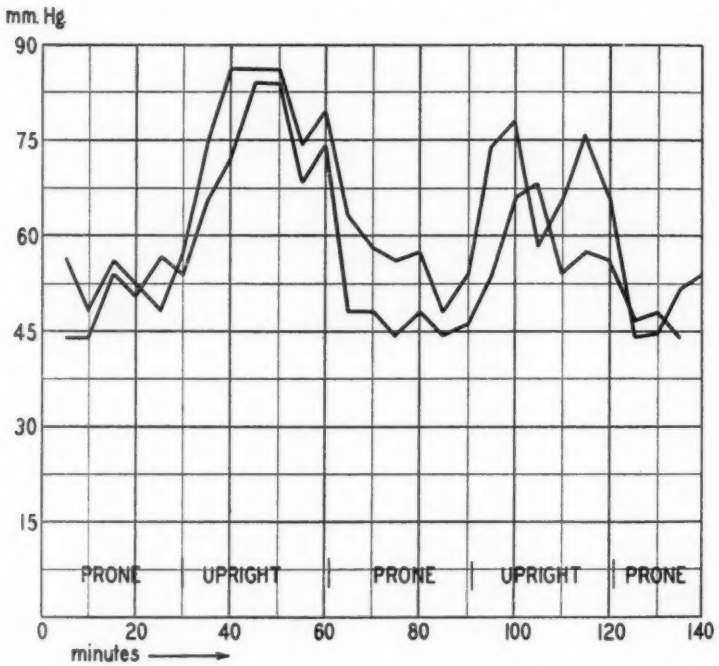
Rabbit no.	Position		Per cent increase
	Prone	Upright sitting	
1	43 mm. Hg	70 mm. Hg	62.8
2	45 mm. Hg	68 mm. Hg	51.1
3	53 mm. Hg	80 mm. Hg	50.9
4	56 mm. Hg	70 mm. Hg	25.0
5	51 mm. Hg	75 mm. Hg	47.1
6	46 mm. Hg	59 mm. Hg	28.3
7	77 mm. Hg	87 mm. Hg	13.0
8	73 mm. Hg	94 mm. Hg	28.8
9	61 mm. Hg	97 mm. Hg	59.0
10	84 mm. Hg	105 mm. Hg	25.0
Mean	59 mm. Hg	81 mm. Hg	39.1

TABLE III

The Effect of Posture on the Systolic Blood Pressure in Hypertensive Rabbits

Rabbit no.	Position		Per cent increase
	Prone	Upright sitting	
1	85 mm. Hg	130 mm. Hg	52.9
2	99 mm. Hg	124 mm. Hg	25.3
3	117 mm. Hg	165 mm. Hg	41.0
4	122 mm. Hg	149 mm. Hg	22.1
5	115 mm. Hg	125 mm. Hg	8.7
Mean	108 mm. Hg	139 mm. Hg	30.0

Both normal and hypertensive rabbits showed a consistent elevation of systolic blood pressure when placed upright in glass jars. This elevation averaged 39.1 per cent in the case of normal rabbits (Table II) and 30.0 per cent in those that were already hypertensive (Table III). The elevation was sustained as long as the rabbit was kept in the upright position and it could be reinduced intermittently (Text-Fig. 2). No significant change in heart weight was observed, when the animals were sacrificed, in the rabbits kept in upright position



Text-Fig. 2. Systolic blood pressure in two rabbits kept alternately in prone and upright sitting positions.

as compared with the controls. Therefore it cannot be claimed that the hypertension produced was of sufficient duration or intensity to lead to cardiac muscular hypertrophy.

The exact mechanism by which the blood pressure is altered by the procedure employed is not obvious. In addition to the altered dynamics of circulation introduced by the abnormal position there are other factors which must be considered. These include excitement, retention of body heat in the glass container and external pressure to the soft abdominal parts with its secondary effects on respiratory

movements. All of these may have participated in some measure in producing the rise in blood pressure. Simple mechanical elevation alone is evidently insufficient to produce sustained hypertension. The blood pressure of anesthetized rabbits, fastened by their extremities on animal boards, was measured by direct cannulation of the carotid arteries. When the board was tilted at an angle of 75 degrees, elevating the head of the rabbit, only a slight transient rise of pressure was usually elicited; the rise persisted for a few minutes and was followed by a fall, frequently to below the original level.

The initial excitement attendant on manipulating the rabbits into the jars probably did not play an important rôle as the rise in pressure persisted during the entire period of postural elevation. During most of this period the rabbits were at rest or even asleep. Increased retention of body heat was a more conspicuous feature. The glass jars became quite warm. The ears showed evidence of increased circulation; they usually became very warm and both arteries and veins stood out very prominently on transillumination. However, change in body temperature alone cannot be held accountable for the observed increase in pressure. Rabbits kept in the glass jars in a prone position showed no elevation of pressure. Moreover the rapid rise that followed when the jars were subsequently placed vertically indicated that the change in posture was the decisive factor.

Discussion

The only demonstrable difference between animals kept in an upright sitting position and the controls was in the level of blood pressure. Since high blood pressure is recognized as a predisposing factor in the development of intimal plaques in man, one is led to offer this as an explanation for the increased deposition of intimal lipids. As already pointed out, in cholesterol-fed rabbits the blood pressure is of less obvious significance in the production of intimal deposits. The marked retention of this material in the body fluids is generally considered to be the chief cause of its eventual penetration into arterial walls. Even under conditions of normal pressure in the systemic arteries and in the pulmonary circulation where the intra-arterial tension is considerably lower, lipid deposits occur with cholesterol retention. However, the blood pressure must also be of importance, even in the rabbit, since the veins and very small arteries are seldom involved. There is thus good reason for believing that in hypertensive rabbits the atheromatous process is intensified. The limited degree of elevation of pressure may cast some doubt on the validity of this suggestion, but no other plausible theory can be advanced. If it is true, the experimental

rabbit lesion has one more feature in common with the spontaneously occurring human disease, namely, it is influenced by blood pressure levels.

Because of the obvious difference in the way the rabbit metabolizes ingested cholesterol as compared to man, it has been seriously questioned whether the arterial lesions thus produced have any relation to human arteriosclerosis. This objection is undoubtedly sound since the underlying mechanism which causes marked retention of this material in the tissues and fluids of the rabbit need not obtain in man. Nevertheless, this does not exclude the possibility that the pathogenesis of the arterial lesions themselves may be the same in both species. The histologic resemblance of the lesions in the rabbit to early human lesions is striking. The tendency of the lesions to be localized at the orifices of arterial branches is common to both species. If, as the data presented indicate, high blood pressure promotes the development of the lesions in rabbits, then the likelihood that they are analogous to the human lesions is further increased.

The findings reported here do not shed much light on the manner in which hypertension may accelerate intimal deposition of lipids. The prevalent explanation is that high pressure injures the artery at especially vulnerable points and that injury favors the deposit of lipids. The acute nature of the process in these experiments rules out injuries of the type that are slow or gradual in development. In a previous report an alternative explanation has been offered for the focal character of lipid deposits in arteries, namely, the involved areas represent parts of relative fixation or immobility to which lipids converge from the adjacent intimal tissue. According to this view, the influence of hypertension would be to increase the rate of penetration of lipids into the intima as a whole. The rapid effect of even moderate degrees of hypertension in the rabbit would support this latter interpretation.

The observations of Dill and Isenhour [12] establish an even closer relationship between hypertension and the formation of atheromatous plaques in the aortas of rabbits. Apparently, even without supplementing the diet with cholesterol, lipid deposits in the intima frequently form in hypertensive rabbits. These lesions were not observed, however, when the hypertensive state had lasted less than 6 months. When present, they were for the most part quite small and limited to the arch of the aorta or to the orifices of the intercostal arteries. There is little doubt that in the experiments reported here, the cholesterol feeding played a decisive part in the rapid development of large confluent lipid deposits.

Summary

The systolic blood pressure of rabbits placed in upright sitting position in glass jars is significantly elevated. This elevation persists during the entire period of postural change, but the blood pressure returns to normal when the rabbits are removed from the jars. This elevation of pressure occurs not only in normal rabbits but in those previously rendered hypertensive by unilateral cellophane perinephritis followed by contralateral nephrectomy.

Rabbits fed high cholesterol diets and placed in the upright sitting position for 5 hours daily develop more abundant intimal deposits of lipids in their aortas than rabbits fed equal amounts of cholesterol but not subjected to postural change. The intimal lesions develop more quickly, are larger and tend to be more widely distributed over the entire aorta, including the abdominal portion.

It seems reasonable to conclude that even moderate degrees of elevation of blood pressure facilitate the passage and retention of lipids in the aortas of cholesterol-fed rabbits.

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DESCRIPTION OF PLATE

PLATE 36

FIG. 1. Photograph of rabbits' aortas showing distribution of intimal plaques. The lipid is stained with Sudan IV and photographs dark gray in contrast to the lighter lipid-free areas. The two aortas on the left are from rabbits fed cholesterol for 8 weeks and maintained in upright sitting position for 5 hours daily. The two on the right represent the maximum and minimum deposits seen in rabbits fed cholesterol for 12 weeks but not subjected to postural change. There are deposits of greater extent in both thoracic and abdominal portions of the two aortas on the left.

1

INTERSTITIAL CELL GROWTHS OF THE TESTICLE *

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The differentiation between hyperplasia and neoplasia in respect to certain rare tumors such as those of interstitial cells is difficult chiefly because of lack of familiarity with the variations of the normal cells. We report two cases of hyperplasia of interstitial cells and two cases of local tumor of interstitial cells. The criteria for the classification of these cases are based on examination of a series of 370 other testes in our files. Since the purpose was to obtain a working knowledge of the variations in the number and distribution of interstitial cells, special sections were not made. Each testis was graded for the following items: number of interstitial cells, atrophy, spermatogenesis. The number of interstitial cells was marked as one to four plus. Four plus was considered high normal or questionable hyperplasia. Tubular atrophy was graded similarly. One plus indicated slight atrophy of tubules; four plus, complete atrophy. Early thickening of the basement membrane without other evidence of atrophy was noted as such rather than as atrophy. The degree of spermatogenesis was indicated as normal, slightly or markedly diminished, and absent. The amounts of fibrosis of stroma, inflammatory reaction, or vascular disease were described. We did not study the interstitial cells for histologic variations except to note whether or not they corresponded to the so-called normal. Unusual distribution of the cells was noted. Marked local variations were described rather than graded, as, for example, spotty complete atrophy in an otherwise normal testis. From the statistical point of view, the data were inconclusive. One to three plus interstitial cells were about evenly distributed among the testes with one to four plus tubular atrophy; of 16 testes with four plus atrophy, 4 had three plus interstitial cells and 2 had four plus interstitial cells. Of the testes without spermatogenesis, there were more with few interstitial cells than otherwise.

An abnormality in the number of interstitial cells was classified as: (1) hyperplasia—an increase of interstitial cells between the tubules without destruction or displacement of the tubules beyond the limits of the tumor; (2) local tumor—a discrete nodule or group of interstitial cells locally replacing or displacing the seminiferous tubules; (3) malignant tumor—increase of interstitial cells with anaplasia, destruction of tubules and metastases.

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The Interstitial Cells in the Normal Testis

The interstitial cells are arranged in compact groups in spaces between the tubules [42] without a consistent relationship to other structures. They are usually polyhedral, sometimes with short processes, and they vary from 14 to 21 μ in diameter. The nucleus is relatively large and spherical, often wrinkled, contains one or two large nucleoli and numerous coarse granules. Binucleate cells are not uncommon [42] and multinucleate cells with as many as thirty nuclei have been described.[43] The two striking characteristics of the cytoplasm are brown granules, thought to be waste pigment (lipofuscin), and crystalloids (of Reinecke). The latter are rod-shaped bodies of unknown composition with rounded or pointed ends,[42] which are polychromatous, staining red with safranine, violet by the method of Bizzozero, red or green by Benda's method.[22] They are not always present, but when numerous or large are quite striking.[42] Less conspicuous constituents of the cytoplasm are the mitochondria and highly refractile granules, some of which are neutral fats and lipoids. Suitable technics bring out an attraction sphere with round or rod-shaped centrioles near the nucleus and Golgi apparatus around the sphere.[42]

The number of interstitial cells varies with age. They are few during intrauterine life and in the first postnatal year.[44, 57] The density of the interstitial tissue, including the interstitial cells whose appearance is unlike that of the adult,[70] decreases as the growth of the tubules increases at the seventh month in utero.[18, 33] The cells remain relatively few in number until puberty, when they are more numerous.[28] From puberty to maturity, there is an increase in the number of interstitial cells.

There is no uniformity among different species of animals as to the number of interstitial cells, their time of appearance, and cycles of growth,[2, 10, 33, 66] although in cats the development of interstitial cells is similar to that in man.[36]

In the normal adult testis, the number of cells has not been studied with the same care as the morphology. Few actual counts have been made and most of the data are based either on general impressions or on calculations.[30, 60] Sand and Okkels [61, 62] have pointed out that the possible limits of normal in regard to the number of interstitial cells surpass what is generally described as the normal histology. They studied testes from 39 castrated sexual offenders and from 33 individuals between the ages of 20 and 70 years who had died suddenly because of accident or suicide. Only 8 of the 33 men in the second group had so-called normal testes. Teem [74] found the individual variation greater in middle age than in young persons. He also noted a

tendency toward clumping of cells after middle life. In our group of 279 testes from persons of known ages, associated with a wide variety of lesions, 63.5 per cent had a normal number and distribution of interstitial cells. In 29.2 per cent the cells were either absent or extremely rare and in 7.3 per cent they were very numerous, possibly in some instances abnormal.

The Relationship of Interstitial Cells to Hormonal Influences

The development of tubules and interstitial cells in inverse ratio at different periods of intrauterine life in man has been linked with the concentration of anterior pituitary-like hormone in maternal and fetal blood. Thus the active growth of the tubules as compared with the interstitial cells at the seventh month of gestation is accompanied by an increase in maternal anterior pituitary-like hormone, and the decrease in this substance by 50 per cent and 25 per cent in maternal and fetal blood, respectively, at the ninth month is associated with the reverse relations of tubules and interstitial cells.[18, 33] Hyperplasia and local tumor of the interstitial cells have been associated with developmental abnormalities of the genitalia, precocious puberty, and tumors of the testis and pineal gland (Tables I and II).

There is much information relative to the interrelationship of interstitial cell growth and activity and certain internal secretions. The most obvious fact in this connection is the well-known seasonal variation in interstitial cells in certain animals. Different interpretations can be drawn from the experiments concerning the effect of anterior pituitary hormone on interstitial tissue.[11, 20, 23] Prolonged injection of prolan for an unstated period in infant and adult mice and rats has been shown to increase the number of interstitial cells, especially in the region of the rete, while it caused only slight atrophy of the tubules.[35] It was not determined whether prolan A or B was responsible. In the mouse and rat, the anterior pituitary stimulates proliferation of interstitial cells.[11] Hyperplasia with some tendency toward neoplasia has been found about 2 months after injection of 3 mg. doses of triphenylethylene in Strong A strain of mice, 4 to 7 weeks old.[6] The estrogen was administered weekly. The neoplastic cells were characterized by very acidophilic cytoplasm, large nuclei and nucleoli, and mitotic figures. An interesting finding was focal hematopoiesis among the tumor cells. In one mouse treated for 52 weeks, the new growth extended through the capsule into the connective tissue of the spermatic cord and metastasized to the upper pole of the right kidney. An interstitial cell tumor was produced in

TABLE I
Interstitial Cell Hyperplasias

Author	Year	Diagnosis	Age in years	Size of testicle	Size of tumor	Surgery or autopsy	Unilateral or bilateral	Character of tumor	Involvement of adjacent structures	Metastases	Mitoses	Remarks
Stroebe[72]	1897	Hyperplasia; pseudohermaphrodismus masculinus internus	63	"Bean-shaped"		Autopsy	Bilateral	Groups; bands; macroscopically visible islands surrounded tubules				Testicles on either side of elongated uterus with well developed round ligaments and tubes
Pick[53]	1905	Hyperplasia; internal masculine pseudohermaphrodite	38	3.5 x 2 cm.	Multiple; largest, 1 mm. in diameter			Groups; strands, nodules; diffuse sheets over-growing the tubules; tubules embedded in tumor; largest mass 1 mm. in diameter				
Dürck[19] (Case 1)	1907	Hyperplasia	25	1.8 x 1 cm.; 7 gm. with epididymis		Autopsy	Bilateral	Sheets of cells between atrophic tubules				
Dürck[19] (Case 2)	1907	Hyperplasia	64	Hazelnut-sized		Autopsy	Bilateral	Cells, large in size, increased between the tubules				Testes in peritoneal cavity; pattern suggestive of polymorphous pigment cell sarcoma
Dürck[19] (Case 3)	1907	Hyperplasia	45	Small		Autopsy	Bilateral	Intertubular masses				
Dürck[19] (Case 4)	1907	Hyperplasia	43	Right: larger than walnut size; left: small		Autopsy	Unilateral (right)	Seminiferous tubules had almost entirely disappeared; cell-poor bridges of fibrillary tissue with intervening spaces entirely filled by polygonal cells with vesicular nuclei			Atypical	Resembled sarcoma; left testicle: diffuse fibrous orchitis without interstitial cells

TABLE I (Continued)
Interstitial Cell Hyperplasias

TABLE I (Continued)
Interstitial Cell Hyperplasias

Author	Year	Diagnosis	Age in years	Size of testicle	Size of tumor	Surgery or autopsy	Unilateral or bilateral	Character of tumor	Involvement of adjacent structures	Metastases	Mitoses	Remarks
Orsós [45]	1931	Hyperplasia in pseudo-hermaphrodite female	44	Nut-sized		Surgery	Bilateral	Increased interstitial cells between the tubules which are partially hyalinized; a few degenerated spermatogens present; many cells smaller than normal; adenomatous pattern in same				Testicles in hernial sac; epididymis present as well as cord, latter ending blindly and continuing as cord leading to vagina (blind); uterus and adnexa present
Grynfeltt, Truc and Guibert[24]	1933	Hyperplasia	56			Surgery	Unilateral	Cords; masses compressing tubules, perivascularly	(1) Walls of tubules; (2) center of fibrous cord replacing seminiferous canal	None	(Amitoses)	Testicle 2 cm. above pubis
Le Chuiton, Prade, Berge and Pennaneac'h[39]	1935	Adenoma	43			Autopsy	Unilateral	Seminoma with hyperplasia of interstitial cells; seen between the tubules and among the seminoma cells; interstitial cells are glycogen-free in contrast to seminoma cells	None	None	Frequent	At site of seminoma-metastases there were no interstitial cell metastases; opposite testicle, normal; no adenoma of interstitial cells present
Cleghorn, Hyland, Mills and Linell[14]	1938	Hyperplasia of interstitial cells; pinealoma	23	Atrophic		Autopsy	Bilateral	Complete atrophy of tubules; hyperplasia of interstitial cells				Pinealoma with invasion of brain stem and hypothalamic region; internal hydrocephalus
Warren and Olshausen	1942	Hyperplasia	46	6.5 x 5.0 x 2.5 cm.	Same as testis	Surgery	Bilateral	Closely packed cells between the tubules	None	None	None	X-radiation postoperatively; urine after operation positive for testosterone and negative for estrin
				4.0 x 3.0 x 2.5 cm.	Same as testis	Surgery		Moderate number of cells between the tubules; in sheets in the epididymis	Epididymis	None	Occasional	This testis removed 3 mos. after the other; hyperplasia in testis, greatest near rete
Warren and Olshausen	1942	Hyperplasia	51	3.5 x 2.0 x 1.8 cm.	Same as testis	Surgery	Bilateral	Reddish brown; increase of cells between tubules	None	None	None	

TABLE II
Interstitial Cell Tumors

Author	Year	Diagnosis	Age in years	Size of testicle	Size of tumor	Surgery or autopsy	Unilateral or bilateral	Character of tumor	Involvement of adjacent structures	Metastases	Mitoses	Remarks
Chevassu[12]	1906	Tumor	27	Small hen's egg	Replaced entire testicle	Surgery	Unilateral	Lobules separated by connective tissue strands; histologically benign; tumor limited by fibrous shell containing a few degenerated tubules	None	None		Alive 22 months postoperatively; ectopic
Kaufmann[21] (Case 1)	1907	Tumor	30	(a) 210 gm., 9 cm. long, 1 cm. in diameter; (b) 140+ gm., 9 cm. long	Entire testicle excepting subalbugineal area	Surgery	Bilateral	Lobules separated by connective tissue; seminiferous tubules only beneath tunica albuginea; largest lobule is cherrystone-sized	None			See next case
Kaufmann[21] (Case 2)	1908	Tumor	34	150+ gm., 9 cm. long		Surgery	Unilateral	Similar to above	None			Other testicle, left *in situ*, was voluminous; author considered all three (including previous case) probably benign, although polymorphism of cells was considered suggestive of malignancy
Rowlands and Nicholson[58]	1929	Adenoma	9	5 x 4 x 3.5 cm. (5× right testicle)	Almost entire testicle	Surgery	Unilateral	Cords and solid sheets; capsule			None	Precocious puberty (onset stage, 6 to 7 years); some regression of pubertal signs 1 year later
Slanina[68]	1930	Interstitial cell tumor	45		Hen's egg; 10 cm. long., 94 gm.	Surgery	Unilateral	Round cells; numerous vacuoles in protoplasm				(Meager information)
Pana[50]	1931	Interstitioma	16	Same as tumor	28 gm., 6.2 x 3.1 x 2.9 cm. (includes tumor, connective tissue and periphery)	Autopsy	Unilateral	Uniform mass with some connective tissue strands; tubules at periphery with connective tissue				Right testicle, 3.5 gm.; small tubules; interstitial cells abundant with some small nodules
Stewart, Bell and Roehlke[71]	1936	Interstitial cell tumor	5	Maximum transverse diameter, 2.8 cm.	1.8 x 1.5 x 1 cm.	Surgery	Unilateral	Not sharply demarcated; cords and solid sheets				Precocious puberty at 4 years with small tumor, 1 cm. in diameter, in testicle; pituitary, pineal, adrenal reported normal; large testicle considered due to tubules rather than to tumor

TABLE II (Continued)
Interstitial Cell Tumors

Author	Year	Diagnosis	Age in	Size of	Size of	Surgery or	Unilateral or	Character of	Involvement of adjacent	Metastases	Mitoses	Remarks

TABLE II (Continued)
Interstitial Cell Tumors

Author	Year	Diagnosis	Age in years	Size of testicle	Size of tumor	Surgery or autopsy	Unilateral or bilateral	Character of tumor	Involvement of adjacent structures	Metastases	Mitoses	Remarks
Jemerin[28]	1937	Interstitial cell tumor	35	6.5 x 6 x 3.5 cm.	Entire testicle except small area at lower pole	Surgery	Unilateral	Anastomosing strands, nests, or broad sheets	None	[illegible]	None	Area at pole contained atrophic tubules without interstitial cells
Budd[9] and Hunt and Budd[27]	1937 1939	Interstitial cell tumor	42	5 x 3.5 x 3 cm.	2 x 2 x 2.5 cm.	Surgery	Unilateral	Elongated or anastomosing columns; capsule penetrated in some places		None		Tubules outside capsule were atrophic; gynecomastia said to regress following removal of testicle; urine and tumor negative for prolan; 20 mouse units of estrin in 1.73 gm. of tumor; positive Zondek reaction equivalent to 1000 units of luteinizing hormone per liter of urine
Braun[7]	1939	Tumor	45			Autopsy	Unilateral tumor; bilateral hyperplasia	Testicle filled by cells up to tunica albuginea				Left testicle shows moderate interstitial cell hyperplasia; patient died of carcinoma of cardiac end of stomach
Huffman[26]	1941	Adenoma	6	2× normal; 5 cm. in diameter	Entire testicle grossly	Surgery	Unilateral	Almost completely encapsulated; irregularly shaped alveolar masses; nuclei round or elliptical; evenly distributed chromatin; large nucleolus	Capsule where tumor does not fuse with albuginea	None; seen 2 years later (hair and gynecomastia still present)	None	Staining property of fat resembles interstitial cells more than adrenal cortical cells; Ponceau stain for androgenic cortical adrenal cells negative; frozen section for anisotropic doubly refractile lipoids negative; deposits of calcium throughout testis; patient had moderate bilateral gynecomastia; Friedman test negative; pubic hair present
Fiahlo[21]	1941	Tumor	44		2.0 x 2.0 x 1.5 cm.	Surgery	Unilateral	Well defined fibrous capsule; polymorphic interstitial cells; contain one nucleus; cytoplasm contains lipoids and pigment	None	None	None	Benign hydrocele accompanied the testicle
Warren and Olshausen	1942	Tumor	30	4.5 cm. in diameter	2 cm. in diameter	Surgery	Unilateral	Brown-yellow to translucent gray-white in color with punctate gray foci; tumor composed of closely packed interstitial cells	None	None	Present	No seminiferous tubules at site of tumor

one mouse of this strain, 30 days old, by weekly subcutaneous injection of 0.05 mg. of estradiol benzoate.[25] Shimkin, Grady and Andervont [65] implanted 4 to 6 mg. cholesterol pellets, containing 10 to 25 per cent of stilbestrol, subcutaneously into 107 strain C male mice, 6 weeks to 3 months old. Three to 5 months after the implantation of the stilbestrol pellets, the mice developed atrophy of the seminiferous tubules, almost complete lack of spermatogenesis and diffuse hyperplasia of the interstitial cells. Thirteen tumors (nodules of interstitial cells) developed in the 11 months after the implantation of pellets. Mitoses were remarkably infrequent.

Hyperplasia of Interstitial Cells in Relation to Spermatogenesis

The infrequency of hyperplasia of interstitial cells described in testes with normal spermatogenesis seems significant, if true. In the group of 33 men from 20 to 70 years who died suddenly, hyperplasia of interstitial cells was found in 2 persons and an unusually large number of cells in 5 others.[61, 62]

It has often been observed that interstitial cells are increased in conditions associated with diminished spermatogenesis. Many writers have described hyperplasia of interstitial cells with atrophy of seminiferous tubules in chronic debilitating diseases as leprosy, syphilis, tuberculosis, carcinoma, pernicious anemia [16, 28, 78] and chronic alcoholism.[78] The increase in interstitial cells described in acute infections, if constant, would be extremely interesting. Cordes [16] studied the testes of 36 patients dying from acute illnesses and found an increase in interstitial cells in 13 of these cases. From a study of 58 persons dying of acute disease, Kyrle [38] concluded that the factor determining the occurrence of hyperplasia of interstitial cells was an injury to the parenchyma sufficiently intensive or long-lasting to produce at least a cessation of spermatogenesis. Sehrt [64] distinguished between the hyperplasia of interstitial cells seen in generalized acute conditions and that secondary to carcinoma. He found that with extratesticular carcinoma the interstitial cells were present in large masses up to 1000 cells, which he claimed is much greater than is found in other wasting diseases. Collins [15] found only an insignificant difference in testes of 173 patients with, and a similar number without, carcinoma, and the same was true in a large group of mice.

Abnormality of interstitial cells has occasionally been associated with carcinoma primary in the testis. Pace [47] and Pace and Cabot [48] reported 3 examples of carcinoma of the testis in a group of undescended testes. The third case is of unusual interest: two pathologists

thought the testis showed adenocarcinoma, while a third regarded it as extreme tubular hyperplasia. Similarly, the increased interstitial cells were regarded as hyperplastic, premalignant and malignant, respectively.

The interstitial cells may be more numerous in the cryptorchid than in the normal testis,[24, 28, 53, 67] but this is not always so. The abnormally placed testis naturally varies with the age of the individual, and interstitial cells are said to be rare in the immature ectopic testis.[22] Among 24 unilateral undescended testes, all without spermatogenesis, hyperplasia of the interstitial cells was reported in only two: (1) a male of 45 years, the testicle being intra-abdominal (without carcinoma), and (2) one with carcinoma.[47, 48]

No relationship was demonstrated between disease of the prostate, benign or neoplastic, and the number of interstitial cells in 504 autopsies studied by Teem.[74] He did find, however, that persons over 69 years of age with hypertrophy of the prostate had relatively fewer interstitial cells than those in this age group with normal prostates.

In our control group of 370 testes, there was no significant relationship between the interstitial cells and the activity of the tubules, the degree of inflammation or the amount of vascular change, which is at variance with the general impression that hyperplasia is accompanied by atrophy of seminiferous tubules. Of the testes of patients with ages known, 21.9 per cent were from persons dead of primary extratesticular malignant neoplasms; 7.7 per cent had primary testicular malignant neoplasms and 1.7 per cent had teratoma of the testis. These did not differ from the others. There were no cases of certain hyperplasia in this control series. Not quite one-third of the testes (111 of 370) showed marked atrophy. Of these 70 cases of known age, only 18.6 per cent had interstitial cells in the upper normal group or possibly slightly above. Of the remaining atrophic testes, about one-half (40 per cent) had interstitial cells in normal numbers and about one-half (41.4 per cent) had few or no interstitial cells. Also, 56 per cent of testes with slight spermatogenesis contained a normal number of interstitial cells. Twenty-seven testes showed marked inflammation, and the interstitial cells were normal in 70 per cent of these. Interstitial cells were below normal in number in our few cases of cryptorchid testes.

Artificially imposed conditions which cause a decrease in spermatogenesis also stimulate proliferation of interstitial cells; *i.e.*, vasectomy, partial castration, testicular graft, changes in environmental temperature, x-ray and radium irradiation,[15, 38] dietary deficiency, ingestion of drugs and administration of glandular extracts.[15]

In animals, an increase in interstitial cells frequently,[66] but not always,[7] is found roughly proportional to diminished testicular tubules whether from disease or subnormal development, as in hybrids.[55] Multiple resections of portions of testes, up to a certain point,[36] variation in nutrition,[36] x-ray therapy,[36] intra-abdominal transplantation,[8, 36] and acute inflammation induced by intratesticular injection of horse serum 2 weeks after a subcutaneous injection,[32] all lessen spermatogenesis and cause increase in interstitial cells. However, the degree of hyperplasia of these cells following x-ray irradiation of testes of dogs seemed out of proportion to the effect on the tubules.[38] In rats 21 days of age or older, the interstitial cells were not affected by a diet deficient in vitamin E for a period of from 20 weeks to 9 months. There was atrophy and necrosis of the spermatic cells, while the Sertoli cells remained unchanged.[29]

Hyperplasia of interstitial cells does not necessarily carry any special morphologic variations. Extratesticular cells may be more common in hyperplasia, although this should not be considered an abnormal feature.[65] Okkels and Sand [45] described unusually large extratesticular cells, 20 to 50 μ in diameter, and nuclei rather poor in chromatin with one large nucleolus. The cytoplasm stained more deeply, crystals were absent and only very slight pigment was seen. Spangaro [70] stated that interstitial cells may pass into the hyaline basement membrane, and thence into the atrophic tubules. Common sites for interstitial cells outside the testis are the body and tail of the epididymis,[4, 42, 56, 76] and along arteries intimately associated with nerves near the corpus Highmori.[1, 5, 45]

We have collected from the literature 10 cases of hyperplasia of interstitial cells in man. These and the 2 cases we report are presented in Table I. The case reported by Le Chuiton, Prade, Berge and Pennaneac'h [39] was classified as adenoma, but we feel it represents hyperplasia, since the interstitial cells were scattered among the cells of a seminoma. The opposite testis was normal. There is only 1 among these 12 cases which might be considered to be hyperplasia in an otherwise normal testis. This is Dürck's [19] third case. Three testes were from hermaphrodites or pseudohermaphrodites and two others were undescended. Atrophy, apparently fairly extensive, was present in three testes. Hyperplasia in 2 cases was associated with tumor. Cleghorn, Hyland, Mills and Linell [14] reported a case of pinealoma with invasion of brain stem and hypothalamus region. The tubules of both testes were completely atrophied and there was hyperplasia of interstitial cells. All of the testes were normal in size or small, except for three which seemed slightly enlarged. Inflammation was

extreme in one testis [19] and prominent in one other (our case no. 2). Extratesticular interstitial cells are reported in only 2 cases; that of Grynfeltt, Truc and Guibert [24] from a cryptorchid, and our case no. 1.

These tabulated cases of hyperplasia, while not completely representative of the literature, are important because of the clarity of presentation and nature of the material. The review of our own cases suggests that uncomplicated hyperplasia is extremely rare. A final decision on the significance of association of hyperplasia and other abnormalities must await more exhaustive study of the testis in experimental and pathologic conditions.

Local Tumor vs. Malignancy of Interstitial Cells

Local tumors collected from the literature are presented in Table II. All of these patients were under 45 years and three of them were under 10 years. These last exhibited precocious sexual development. Stewart, Bell and Roehlke [71] considered the enlargement of the testis in their case as due to premature maturation of the seminiferous tubules rather than to the interstitial cells, although the tumor occupied nearly one-fourth of the testicle. These symptoms could not be ascribed definitely to other endocrine glands. One of the children also had gynecomastia. Gynecomastia was present in one adult. Symptoms regressed after removal of the testis in the children. The tumors were for the most part large and in six instances involved practically the entire testis. In three individuals hyperplasia was present in the opposite testis. Five of the tumors were encapsulated. Chevassu [12] and Kaufmann [31] described lobulation of the tumor.

The local tumors described in animals are frequently associated with old age and atrophy.[3, 37, 49, 52, 63] In a series of 59 testes from 48 dogs, for the most part old, Schlotthauer, McDonald and Bollman [63] found 51 rather sharply demarcated interstitial cell masses. Only a very small proportion showed normal tubular structure. Aspermia or atrophy was present in the majority. Among 400 dogs examined by Peyron, Blanchard, Poumeau-Delille and Salomon,[52] 21 had nodular hyperplasia of interstitial cells. Fourteen others had encapsulated tumors; 15 had tumors with more active growth, 2 of which showed change of a malignant type. They reported interstitial cell tumors in horses as well, but these are rare. There was only one hypertrophic nodule and one benign tumor among 275 old animals.

Three cases of malignant interstitial cell tumors in man have been reported: one by Masson and Sencert,[41] the second by de Josselin de Jong,[17] and the third by Masson.[40] These comprise Table III.

Six cases of neoplasia are controversial. Sacchi [59] reported his case

TABLE III
Interstitial Cell Carcinomas

Author	Year	Diagnosis	Age in years	Size of testicle	Size of tumor	Surgery or autopsy	Unilateral or bilateral	Character of tumor	Involvement of adjacent structures	Metastases	Mitoses	Remarks
Masson, and Sencert[41]	1923	Interstitial cell cancer	62	8 x 4 x 4 cm.		Surgery	Unilateral (right)	Tubules only beneath the tunica albuginea; tumor: anastomosing cords, 3-4 cells thick, have invaded the surrounding capsule	None	At postmortem: right groin, right femur, right lung, left lung; external iliac, lumbar, and mesenteric lymph nodes; dura mater	Present	Left testicle: benign; normal number of interstitial cells; between degenerated tubules there are voluminous masses; cord shows normal quantity of interstitial cells; first metastases appeared 4 years after appearance of original tumor and 3 years and 10 months after removal of tumor; died 4 years and 4 months after appearance of original tumor
de Josselin de Jong[17]	1926	Interstitial cell tumor: sarcoma arising from Leydig cells; internal masculine pseudohermaphrodite	42	Child's head	Child's head	Surgery	Unilateral	Center: soft, bloody, necrotic; few islands of cells; at edge, cells in bands, rows, or irregular groups		Yes; location not mentioned	None	Left testicle: intra-abdominal; contains "sarcoma." Right testicle: acorn-sized; at external inguinal ring; tubules poorly developed; interstitial cells in groups, largest measures 2-5 mm.; represents a local tumor
Masson[40] Venning[78]	1942	Interstitial cell tumor	32	5 x 4 x 4 cm.		Surgery	Unilateral	Interstitial cell tumor comprises the corpus Highmori and the atrophic and sclerosed testicular parenchyma; voluminous tumerous nuclei; nodules along venules; early metastases(?)	None	Postmortem: left spermatic cord; liver (7.5 Kg.) (largest nodule, 5 cm. in diameter); lungs, bilateral; dura mater	None	First metastases appeared 10 years after appearance of original tumor and 9 years after removal of tumor; died 10 years after appearance of original tumor; urinary hormone studies (see text)

as teratoma, but Stewart, Bell and Roehlke [71] regarded it as an interstitial cell tumor. Peppere's[51] case of sarcoma of Leydig cell origin was regarded by Jemerin [28] as a possible seminoma. Snellman [69] reported a case as interstitial cell "tumorlike" hyperplasia, but the diagnosis seems doubtful in the presence of many melanin-laden cells in the tumor. Three cases of Stoppato [72] answer the description for seminoma better than for interstitial cell tumor. Three cases offered insufficient data for classification (Table IV): Villata [77] reported a case of teratoma, in which there was a considerable number of interstitial cells. Ciceri [13] mentioned a case of interstitioma. Pitrollfy-Szabó [54] likewise described a case of interstitial cell tumor without details.

Case Reports of Hyperplasia of Interstitial Cells

Case 1

A. O. was 46 years of age. The left testis was removed because of spontaneous enlargement of 3 weeks' duration. The initial pathologic diagnosis was carcinoma of interstitial cells. X-ray treatment was started soon after operation. A total of 3200 r. units was given, 200 r. units over one area daily to anterior and posterior pelvic areas and 2800 r. units to upper abdomen, anterior and posterior. The following factors were used: 200 kv.; 8 ma.; 50 cm. skin target distance. A urine sample taken for estrin and testosterone 5 weeks after operation was positive for testosterone and negative for estrin. The right testis was thought to be somewhat enlarged and was removed 14 weeks after the first operation. Roentgenograms of the chest taken 1 year later showed a questionable shadow at the base of the left lung. X-ray treatment to the pelvis was repeated approximately 2 years after the first operation. The patient is now well, 5 years after removal of the tumor, without clinical or x-ray evidence of disease.*

The left testis measured 6.5 by 5 by 2.5 cm. The tunics and 3 cm. of the cord were grossly normal. The testis was symmetrical and had a normal shape. Section revealed homogeneous yellowish white tissue with slightly increased consistency and indistinct tubular markings. The external aspect of the epididymis was as usual but the lumen was obscured by fibrous tissue. Microscopic sections were stained with hematoxylin and eosin and with iron hematoxylin-Masson green. Atrophy of the tubules was uniform and almost complete. A few swollen, partly degenerated spermatic cells were still present within the tubules. Besides these, there were cells of different appearance, not definitely identified. Because of some characteristics in common with the interstitial cells, the question of invasion of the tubules by these cells was considered, but could not be definitely answered. The intertubular spaces were filled with rather closely packed polyhedral cells. These cells averaged 12.1 by 9.1 μ in size. The extremes measured 18.5 by 14.9 μ and 8.7 by 8.2 μ. A moderate number of cells had two nuclei and an occasional one had three. These were round, staining lightly with iron hematoxylin, and had one or two nucleoli. No mitoses were seen. Fine, deeply staining granules as well as occasional vacuoles were present in the cytoplasm. Some of the granules stained with light green SF yellowish rather than with iron hematoxylin. Interstitial cells were not found in the tunica albuginea or in the epididymis. A moderate number

* We are indebted to Dr. James E. Waters for the material, and to Dr. Clarence N. McPeak for the x-ray studies.

TABLE IV
Interstitial Cell Growths

Author	Year	Diagnosis	Age in years	Size of testicle	Size of tumor	Surgery or autopsy	Unilateral or bilateral	Character of tumor	Involvement of adjacent structures	Metastases	Mitoses	Remarks
Villata[77]	1928	Teratoma	22	Larger than 2× normal			Unilateral	Two types of cells: (1) large round with clear cell-body, (2) cells in cords (prevalent)				Teratoma including interstitial cells
Ciceri[18]	1933	Interstitioma				Surgery						
Pitrollfy-Szabó[84]	1937	Interstitial cell tumor				Surgery						

TABLE V
Local Tumor with Interstitial Cell Hyperplasia

Author	Year	Diagnosis	Age in years	Size of testicle	Size of tumor	Surgery or autopsy	Unilateral or bilateral	Character of tumor	Involvement of adjacent structure	Metastases	Mitoses	Remarks
Warren and Olshausen	1942	Local tumor plus interstitial cell hyperplasia	44	Small (bilateral)	Same as testis	Autopsy	Bilateral	Increased number of interstitial cells between tubules	Tunica albuginea	None	None	Tumor arising from hyperplasia; in some areas seminiferous tubules are present, while in others, they have been destroyed

of lymphocytes were interspersed among the interstitial cells and there were also occasional collagen strands.

The right testis measured 4 by 3 by 2.5 cm. The contour of the testis, epididymis and 2.2 cm. of cord was not unusual. The tunica was slightly thickened by fibrous tissue and there was evidence of old fibrin toward the lower pole. Adhesions obliterated the sac of the tunica vaginalis in part and there was slight extension of fibrous tissue into the testis. This fibrosis was especially marked adjacent to the epididymis. The bulk of the testis approximated normal, but the tubules would not string out appreciably. The epididymis was almost entirely fibrosed. The microscopic sections showed some atrophy, but this was less marked than in the left testis. There was no spermatogenesis and the Sertoli cells were edematous and vacuolated, the nucleus having been displaced to the base of the cell. Interstitial cells were present in moderate numbers between the tubules. Their appearance did not vary greatly from the normal. They were polyhedral. The cytoplasm was finely granular with occasional small vacuoles. The nucleus, occupying one-third to one-half of the cell, was also finely granular. Double or triple nuclei were not unusual and nucleoli varied from one to three. A few interstitial cells were oval or elongated, suggesting primitive mesenchymal cells. Interstitial cells were most numerous in the region of the tubuli recti and sheets of them lay between the coils of tubules in the epididymis. There were a few mitotic figures in these latter sites. An inflammatory reaction with fibroblasts and infiltrating cells, chiefly lymphocytes with a scattering of polymorphonuclears, was fairly conspicuous in the stroma throughout the testis. This reaction was present in about the same degree in the epididymis.

Case 2

A. S. was 51 years of age. A radical excision of the external genitalia was done for carcinoma of the penis following observation over 12 years for multiple sinuses and strictures of the urethra. The left testis measured 3.5 by 2 by 1.8 cm. and the right, 1.7 cm. in greatest diameter. The latter contained somewhat more fibrosis than the left testicle. The portion of the right testis near the globus minor was more yellow and less firm than the rest of the testis. Microscopically, both testes were atrophic, especially the right. In both, there were large aggregates of from 25 to 100 interstitial cells. The average measurements of the cells were 14.7 by 9.1 μ. The extremes of size were 21.9 by 11.2 μ and 9.1 by 7.9 μ. Since the tubules had not been replaced by interstitial cells, the increased number of those cells in both testicles has been regarded as interstitial cell hyperplasia.*

Case Reports of Tumor of Interstitial Cells

Case 3

E. B., 44 years of age, had generalized lymphoblastoma of 5 years' duration. Small doses of x-ray had been given to the lymph nodes in various locations during that time, but the testes were never exposed. At autopsy, both testes were atrophic. There was marked atrophy of seminiferous cells and hyaline thickening of the basement membrane. Large islands of interstitial cells separated the tubules and in part appeared to replace them. The cells extended slightly along the outer sheath of blood vessels into the tunica albuginea. No mitotic figures were present. In view of the character of the cells and the absence of metastases, this growth cannot be considered malignant, although some aggregations of interstitial cells had overgrown focally the seminiferous tubules and destroyed them. The fairest interpretation seemed to be local tumor associated with hyperplasia.

* We are indebted to Dr. Loring H. Raymond for the material of this case.

Case 4

J. R. was 30 years of age. This patient presented himself to his physician complaining of digestive disturbances. On physical examination, a tumor was found in the right testis. The initial pathologic diagnosis was interstitial cell carcinoma. This was said to have been present for 6 weeks. A right orchidectomy was performed. Following the operation, chest films and Aschheim-Zondek test were negative. The patient is now symptomless, 2 years after orchidectomy.

The testis measured 4.5 cm. in greatest diameter. In the upper pole a discrete tumor 2 cm. in diameter differed in color and consistency from the rest of the testis which approximated normal. This tumor was composed of densely packed polyhedral cells averaging 14.9 by 11.6 μ. The extremes measured 23.1 by 18.8 μ and 11.0 by 8.4 μ. The cells differed from the normal interstitial cells in being larger. Their cytoplasm contained densely packed fine acidophilic granules and fine vacuoles. Occasional mitotic figures were seen. There were no tubules within the tumor and those at the periphery were compressed. The remainder of the testis was not unusual.* We consider this a local tumor of interstitial cells.

Discussion of Cases

A study of the tabulated human cases † of hyperplasia, tumor and malignant neoplasia reveals the following:

Age. Fifty-seven per cent of the cases of hyperplasia occurred at or above 45 years of age, while all of the local tumors occurred at or under 45 years of age. Two of the three cases of malignant neoplasms occurred under 45 years of age.

Size of the Testicle. The data for hyperplasia are too indefinite for comparative purposes; however, the testis is usually small. In contrast, fifty-seven per cent of the local tumors were one to two times the size of a normal testicle, while 19 per cent were normal in size or less.

Surgical or Postmortem Material. Fifty-eight per cent of the examples of hyperplasia were obtained at autopsy, while 85 per cent of the tumors were obtained by surgical procedures. The malignant tumors were removed surgically.

Involvement of Adjacent Structures. Of the 28 cases, 3 cases of hyperplasia showed involvement of the adjacent structures; namely, the atrophic tubules, tunica albuginea and epididymis, respectively.

Metastases. Metastases were found in three cases, one of which showed diffuse involvement of the testicle with no tubules remaining. One tumor was surrounded by a capsule, and in the third case, tumor was found along small blood vessels and was suspected of being possibly early metastasis. None of the testicles with solid tumors or so-

* We are indebted to Dr. Earl E. Ewert of the Lahey Clinic for the clinical data.

† One case, which showed histologic characteristics of both hyperplasia and local tumor, was not included in the series for statistics. This case is tabulated separately (Table V).

called hyperplasia presented any metastases. In only one of these were tubules absent, and this patient lived for at least 22 months postoperatively.

Mitoses. Three cases of hyperplasia, one local tumor, and only one of the three malignant tumors showed mitoses.

Ectopic Testicles. Four of the testicles with hyperplasia and one of those with malignant neoplasms were ectopic.

Malignant Neoplasms. One of the malignant tumors occurred in the age group (above 45 years) in which hyperplasia is most common, while the other two occurred in the age group in which local tumor is most common. De Josselin de Jong's case [17] was ectopic. Mitoses, found in one of the malignant neoplasms, were found also with hyperplasia and local tumor. In Masson and Sencert's case [41] as well as in that of Masson [40] the malignant tumor may have arisen from a local tumor, as only a few interstitial cells were seen among the few tubules in the first, and the tumor was compressing the tubules in the second case.

One of Dürck's [19] examples of hyperplasia showed irregular cells and atypical mitoses, and appeared similar to sarcoma.

It is thus seen that, to date, no definite criteria for malignancy can be named, except the presence of metastases. However, it appears that a patient whose testicle contains an increased number of interstitial cells with or without mitoses must be carefully watched for the occurrence of metastases. It is known that a latent period of 9 years may occur between the removal of the original tumor and the clinical appearance of metastases.[40]

The great variation in the descriptions of interstitial cell tumors in man as given by various authors is rather difficult to understand. Among the varied pictures a resemblance to cords of seminiferous tubules and to adrenal cortical malignancies has been described.

Hormones. Hormonal determinations in patients with interstitial cell growths have been few. In the case reported by Masson [40] and Venning,[75] the 24-hour urinary output of gonadotropic and estrogenic substances was 110 and 113 mouse units per day, respectively (slightly elevated above normal). The 24-hour urinary 17-ketosteroid level was elevated fifty times above the normal mean. Three determinations made in the presence of metastases 1 to 2 months before death were evaluated at 980, 1035 and 1040 mg. per day. These are the highest 24-hour urinary 17-ketosteroid determinations ever recorded, including those for adrenal tumors.[75] An hydrolysis of urine revealed androsterone sulfate and androstenone. The serum 17-ketosteroid level was 16 mg. per cent.

Summary

Too little is known of the normal variation of interstitial cells to differentiate clearly pathologic states of these cells. On the negative side, there are certain helpful facts:

1. Large collections of interstitial cells are not necessarily pathologic.
2. Multinucleated cells, and cells in the tunica albuginea and epididymis do not necessarily indicate invasive growth.
3. Cyclic changes in the number of interstitial cells from fetal life to senility tend to correspond inversely with changes in spermatogenesis.

Hyperplasia of interstitial cells as found in chronic diseases, injury, castration, cryptorchidism, changes in environment, ingestion of drugs in man, and exposure to x-ray and radium, transplantation of testicles, administration of glandular extracts (prolan B and estrogens) and serum in animals may be due entirely or largely to the diminished spermatogenesis. Of 29 reported human cases of increase in number of testicular interstitial cells, including 4 cases of our own, 12 were classified as hyperplasia, 13 as local tumor, 1 as local tumor accompanied by hyperplasia and 3 as malignant.

The criteria for this classification have been presented.

Hyperplasia occurs principally at or above 45 years of age in atrophic testicles and is discovered chiefly at autopsy. Local tumors occur predominantly at or below 45 years of age in testicles larger than normal, and are generally discovered during life and removed surgically.

Two of the three malignant tumors occurred below 45 years of age.

No definite criteria for malignancy can be named, except for the presence of metastases. However, if the testicle of a patient contains an increased number of interstitial cells, especially if the seminiferous tubules have been partially or completely destroyed, he should be carefully followed for more than 10 years for the appearance of metastases, if not present before that time.

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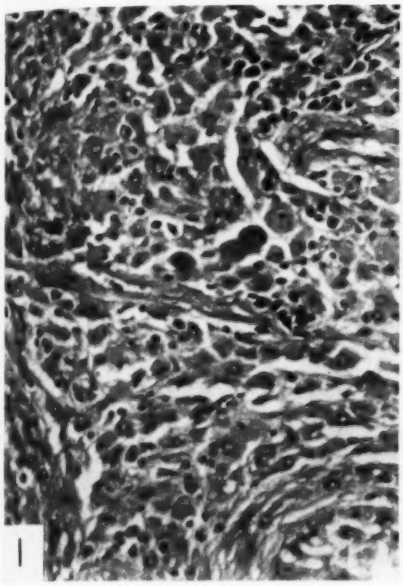
DESCRIPTION OF PLATES

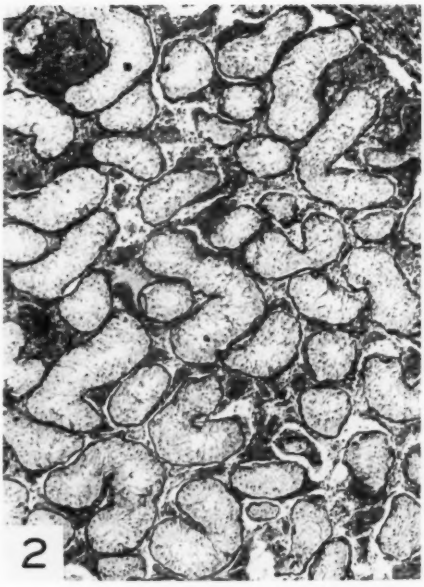
PLATE 37

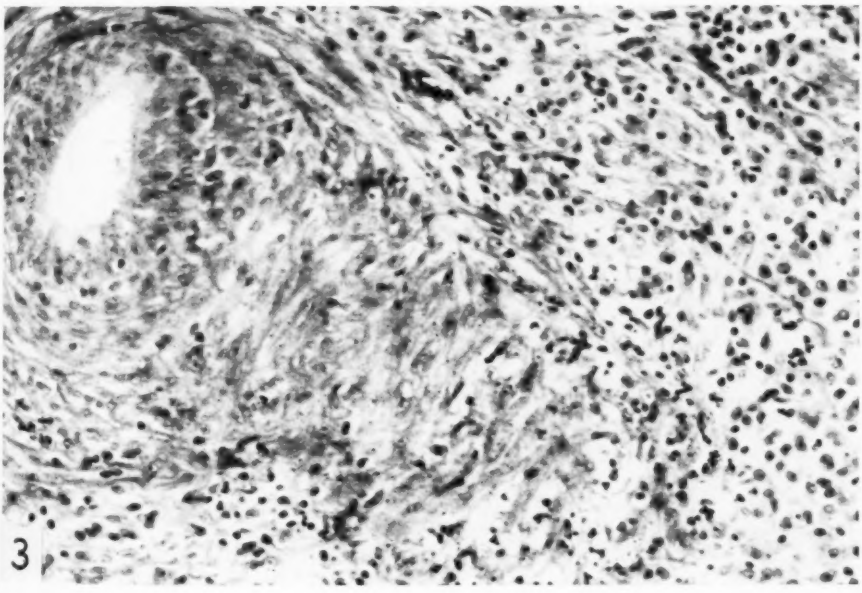
FIG. 1. Case 1. Interstitial cells in first testicle removed. A portion of an atrophic tubule is shown in the lower right corner. × 235.

FIG. 2. Case 1. Atrophic seminiferous tubules and increased interstitial cells in second testicle removed. × 45.

FIG. 3. Case 1. Epididymis of second testicle removed, showing large number of interstitial cells. × 235.







Warren and Olshausen Interstitial Cell Growths of the Testicle

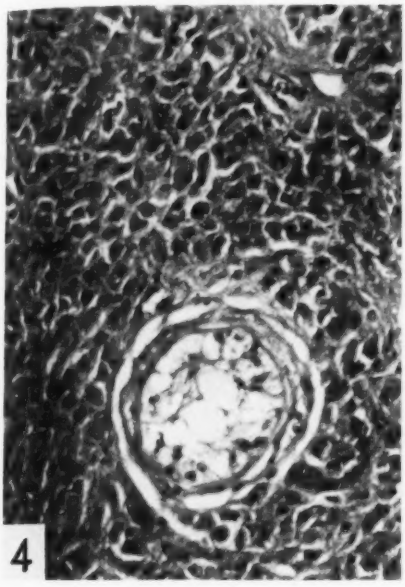
PLATE 38

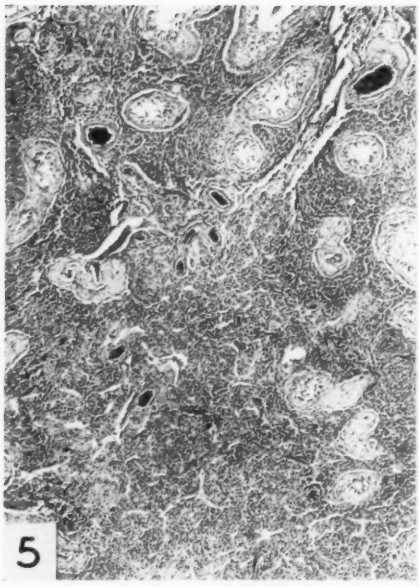
FIG. 4. Case 3. Interstitial cells and atrophic seminiferous tubule. × 235.

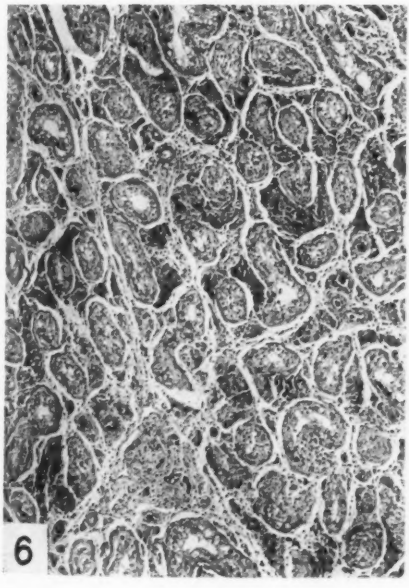
FIG. 5. Case 3. Interstitial cells and atrophic seminiferous tubules. There is a marked diminution in the number of tubules in the lower half. × 45.

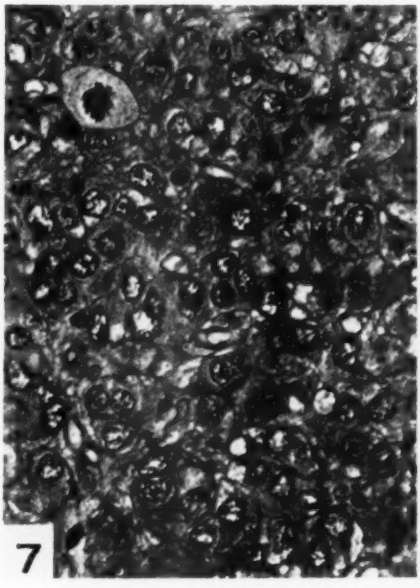
FIG. 6. Case 2. Increased interstitial cells between atrophic seminiferous tubules of left testicle. × 45.

FIG. 7. Case 4. Interstitial cells in tumor with mitotic figure. × 470.









Warren and Olshausen Interstitial Cell Growths of the Testicle

FOCAL GLOMERULITIS IN ELDERLY PATIENTS *

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In a previous report [1] it was established experimentally that severe reduction of functioning renal parenchyma may be associated with proliferative glomerulitis and the hypothesis was suggested that the production of these glomerular lesions was caused by excessive work demand placed upon the glomeruli.

The present study was undertaken to determine the frequency of this type of glomerulitis in persons 60 years of age or older since reduction in the number of glomeruli is a natural accompaniment of senescence.[2]

METHOD

One hundred and seventy-four cases were selected from the autopsy file of The Western Pennsylvania Hospital. All patients were 60 years of age or older. None had diffuse glomerulonephritis or clinical evidence of pyelonephritis. In many instances only one slide was available for study. Only those cases were included in which the sections were technically satisfactory. Where paraffin blocks were available in cases showing severe involvement, additional sections were prepared and stained with azocarmine.

One hundred or more glomeruli were studied in each case and an estimation made of the percentage of glomeruli with inflammatory changes. Altered glomeruli in regions of scarring or of cellular infiltrations were not considered pertinent. Glomeruli which showed pericapsular fibrosis were likewise excluded. The cases were arbitrarily graded according to the incidence of glomerulitis: from 0 to 9 per cent, negative; from 10 to 24 per cent, slight; from 25 to 49 per cent, moderate; and 50 per cent or more, severe.

Autopsy records and clinical charts of the cases with severe involvement were consulted in an attempt to correlate the renal changes with cardiac weights and with the presence or absence of hypertension or uremia.

For a control series, 46 patients between the ages of 17 and 38 years were selected similarly from the autopsy file and the kidney sections studied with the same criteria in mind.

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Results

Description of Glomerular Alterations

In well defined cases even a cursory examination of the sections indicated an increased cellularity of the glomerular tufts (Fig. 1). This abnormality constituted the most frequently encountered alteration. Admittedly, the decision whether the cellularity in any instance is increased or not is arbitrary. However, in this study only those glomeruli were considered of increased cellularity in which the increase in the number of cells did not appear questionable. In reaching this decision an absolute increase in cellularity was differentiated from the relative increase in cellularity caused by collapse of capillaries.

As indicated by the azocarmine stain, the increased cellularity was in some instances caused almost exclusively by an increase in epithelial cells. In other glomeruli principally the endothelial cells were increased in number. However, more often cells of both types were more or less equally increased in number. The distribution of nuclei throughout the glomerulus was not always uniform. "Bunching" or crowding of nuclei as noted in the rat [1] was also seen in a few of the glomeruli. No pyknosis was observed. The nuclei appeared normal. Focal necrosis was not found.

A second notable alteration, evident also on cursory examination, was a more solid and rounded, "simplified" appearance of many of the glomeruli. These glomeruli differed appreciably from sclerotic glomeruli which, although also solid and simplified, were contracted, relatively acellular and possessed obliterated capillaries. The type of glomerulus under discussion was of average size or frequently greatly enlarged, the cellularity increased, and the capillaries clearly discernible, although often empty of blood. The solid appearance of the glomerular tufts was caused by a pronounced increase in cytoplasmic constituents. Both epithelial and endothelial cells were greatly enlarged. The cytoplasmic bodies of endothelial cells frequently occluded capillary lumina and the cytoplasm of epithelial cells was at times even more abundant. The epithelial cells seemed to cement adjoining glomerular loops together and to fill the spaces between them. Thus, the complex outline of the tufts was obliterated and "simplification" produced.

The endothelial and epithelial cytoplasm of the glomerular tufts was frequently of hyaline character and, in azocarmine-stained sections, was a delicate purple or, at times, a very intense, homogeneous dark blue which was continuous with the dark blue-stained substance of

the basement membrane. Only rarely were distinct cytoplasmic granulations seen which corresponded to the hyaline cytoplasmic granulations described by McGregor.[3] In azocarmine-stained preparations the blue-staining cytoplasm of epithelial and endothelial cells often contributed to an irregular thickening of the capillary basement membrane. However, many of the affected glomeruli showed little or no thickening of the capillary basement membrane. Intracapillary fibrillae were not observed.

Proliferation of capsular epithelium was another prominent feature which was found either with or without proliferative changes in the tufts. In a few glomeruli the nuclei of the capsular epithelium presented the appearance of a chain of closely approximated beads (Fig. 2). In addition to hyperplasia there was at times also hypertrophy of the capsular epithelium which assumed cuboidal or even tall columnar forms. These capsular epithelial changes were not associated with pericapsular fibrosis.

An occasional section exhibited one or two glomeruli in which focal adhesions were present between tuft and capsule (Fig. 3). These adhesions were epithelial in character and were not associated with fibrous tissue proliferation in either the tuft or the capsule. All changes were generally greatly exaggerated in and near foci of pyelonephritis.

Incidence of Glomerular Alterations in the Elderly Group and Clinicopathologic Correlation

The proliferative glomerular changes were usually mild in degree. About one-half of the cases showed no significant involvement. In 20 per cent of the 174 cases the glomerular involvement was classified as slight; in 13 per cent, as moderate; while in 30 cases, or 17 per cent, the changes were more extensive and severe. The weight of the heart was indicated in 27 of the latter (permission to examine thoracic organs had been withheld in the other 3). In 16 of the 27 cases there was cardiac hypertrophy (375 to 540 gm.). Many of these patients were hypertensive and some were uremic. The cardiac weights in the other 11 cases ranged between 225 and 350 gm. and although the blood pressure was not charted in all, when recorded it was within normal limits. Similarly, chemical determinations upon the urine and blood showed no significant elevation in these cases. Nephrosclerotic changes were prevalent in most of the cases but were least in evidence in the 11 which showed severe proliferative glomerular involvement and no cardiac hypertrophy.

Incidence of Glomerular Alterations in the Control Group

Only 11 per cent of the controls (5 of 46 cases) showed glomerular changes comparable to those in the preceding series. The most severe changes were found in a white male, 36 years old, who died of hepatic cirrhosis with ascites. The glomerular tufts were smaller than average and showed increased cellularity with pronounced hyalinization of the proliferated cells. They were somewhat similar to the changes described by Horn and Smetana [4] in a case of hepatic cirrhosis.

There were 3 cases with moderate glomerular involvement:

(a) A white male, 28 years old, who died of leptomeningitis and bronchopneumonia. Although the blood pressure was not recorded, the earlier existence of hypertension seems probable in view of a considerable cardiac hypertrophy (425 gm.) in the absence of valvular, or other cardiac defects. (b) A white male, 29 years old, who died of spongioblastoma multiforme. There was subclinical pyelonephritis. The heart weighed 300 gm. (c) A white male, 35 years old, who died of gastric carcinoma with metastases. The blood pressure was 180/130 and the cardiac weight was 350 gm.

The fifth case showed relatively milder glomerular involvement. This was a white women, 32 years old, who died of bronchopneumonia. She had mitral stenosis and the heart weighed 375 gm.

Proliferation of capsular epithelium was surprisingly common (22 per cent) in the control group. However, it was not nearly so pronounced or so widespread as in the older age group.

Discussion

Inflammatory glomerular lesions identical with those found in diffuse glomerulonephritis have been described by McGregor [3] in essential hypertension and by Kimmelstiel and Wilson [5] in hypertension and pyelonephritis. The latter described many of the histologic features found in the present study under the term "alterative" glomerulitis. There are, however, a number of features which are different. According to Kimmelstiel and Wilson [6] the lesion is primarily a degenerative one and is associated with focal necrosis, and adhesions of tuft to capsule are of fibrinous character. Proliferative changes are considered secondary and may even be associated with exudative processes. McGregor described capillary loops occluded by inflammatory monocytes and hyaline fibers in addition to the other features, but made no mention of necrotic foci. She considered hyaline degeneration of epithelium secondary and a sequence to proliferation.

There appears to be little doubt that the glomerular changes described in this paper are of the same type as those described in a previous experimental study [1] and by others.[7-9] Existing differences

in interpretation, emphasis and minor detail are inevitable and understandable. These glomerular lesions have been linked with the hypertensive and the uremic states.[3, 5, 10] Kimmelstiel and Wilson[5] found such lesions in 13 of 56 cases of pyelonephritis. Only 1 of the 13 was not hypertensive. All had renal insufficiency.

Kimmelstiel and Wilson[10] stated that "renal impairment cannot be excluded as an etiologic factor" and also that "in view of the inconclusive character of the evidence, however, we still entertain the possibility of an extrarenal toxin which may produce vascular spasm with coincident renal failure and alterative glomerulitis." The present study indicates that the glomerulitis under discussion is not necessarily associated with either the uremic or the hypertensive state because it has been found with considerable frequency in 11 persons who had neither hypertension nor clinical evidence of renal impairment. The results here presented give added support to the hypothesis that pronounced reduction in functioning renal parenchyma and the resulting excessive work demand placed on the remaining glomeruli is a probable etiologic factor of these glomerular lesions.

An assumption of reduction of renal parenchyma as an etiologic factor of the glomerular lesions in the control group (11 per cent) does not appear valid. The youth of these patients and the absence of scarring or serious active inflammation in the kidneys precludes such assumption. This suggests that the type of glomerular damage under discussion may also be produced by a number of other conditions, among which may be hypertension,[3, 5, 10] chronic pyelonephritis,[5] and possibly others.

One of the puzzling features in differentiating histologically between advanced chronic glomerulonephritis and certain cases of advanced nephrosclerosis (benign hypertension) has been the presence of glomerulitis in the latter which, as McGregor[3] has pointed out, may be identical with that seen in the former. The question has arisen whether there is a superimposed glomerulonephritis in such cases of nephrosclerosis. The recognition of the glomerulitis under discussion as an accompaniment of hypertension, uremia, or senescence seems to offer an attractive explanation to an otherwise confusing picture.

Summary

1. Inflammatory glomerular changes were found in about one-half of 174 patients of 60 years or older who did not have glomerulonephritis or clinical evidence of pyelonephritis. Only 11 per cent of the patients between the ages of 17 and 38 years exhibited such lesions.

2. The glomerulitis was of proliferative character, involving the epithelium of the tuft and capsule and also the capillary endothelium.

Occasional adhesions between tuft and capsule, and hyaline degeneration of epithelium and endothelium were other important findings.

3. The frequency of the glomerulitis was slight in 20 per cent of the cases, moderate in 13 per cent and severe in 17 per cent (30 cases). In 11 of the 30 patients with severe involvement there was no hypertension or clinical evidence of uremia.

4. These findings are interpreted as supporting the hypothesis that the genesis of such lesions in some of these patients is related to a pronounced reduction in functioning renal parenchyma and that it is probably dependent upon excessive work demand placed on the remaining glomeruli.

5. Other conditions may also cause the proliferative glomerulitis under discussion. These may include hypertension and chronic pyelonephritis.

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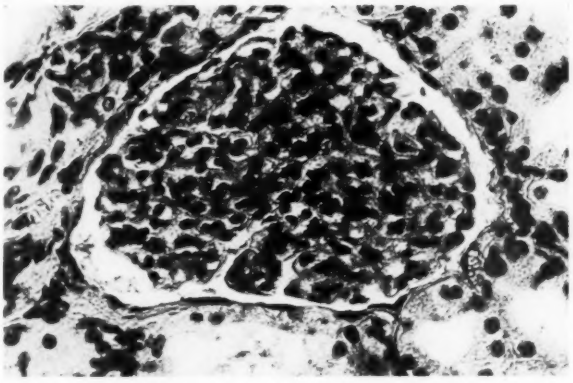
DESCRIPTION OF PLATE

PLATE 39

FIG. 1. Glomerulus showing marked increased cellularity and simplified appearance of the glomerular tuft with a normal capsular epithelium. Hematoxylin and eosin stain. × 290.

FIG. 2. Glomerulus showing proliferation of the capsular epithelium with a beadlike arrangement of the nuclei. The glomerular tuft is normal in appearance. Hematoxylin and eosin stain. × 290.

FIG. 3. Glomerulus showing proliferation and swelling of the glomerular epithelium in the upper left portion of the tuft and an adhesion between the tuft and capsule in the upper right portion of the tuft. Hematoxylin and eosin stain. × 290.



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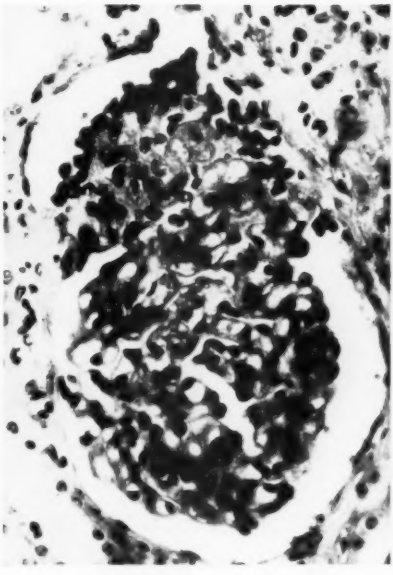

3

Gross and Morningstar Focal Glomerulitis in Elderly Patients

DEVELOPMENT OF MYOCARDIAL NECROSIS AND ABSENCE OF NERVE DEGENERATION IN THIAMINE DEFICIENCY IN PIGS *

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Cardiac failure is well recognized as a part of the syndrome of beriberi in man. The nature of the physiological disturbance leading to such failure has not been well understood and the character of the anatomical changes in the heart itself has been a matter of dispute. Deficiency of the thermolabile portion of the vitamin B complex has long been recognized to be the chief cause of beriberi but, until recently, animal experiments in which a deficiency of this part of the B complex was produced have thrown little light on the cardiac aspects of the disorder.

In several recent reports, however, pathological changes have been described in the myocardium of pigeons,[1] dogs [2] and pigs [3] in which deficiency was produced by feeding autoclaved, or sodium sulfite-sulfur dioxide treated, diets. The changes noted have been attributed to lack of thiamine, both because this has been regarded as the chief or only vitamin of the B complex injured by such treatment of the diet and because improvement followed its administration. Thiamine therapy has also been described as dramatically alleviating the symptoms and signs of cardiac embarrassment in certain cases of cardiac failure in man.[4, 5]

As we have pointed out elsewhere,[6] experiments which depend for the elimination of thiamine on procedures designed to destroy it are open to criticism since, in the process of destruction, other vitamins may be affected as well. Our own studies of thiamine deficiency were not commenced until crystalline vitamins of the B group became available in such quantities and variety that it was possible to secure good growth and development by feeding a vitamin B-deficient diet supplemented only by crystalline vitamins.[7] Although it must be recognized that our basal diet, even when supplemented in this way, may still be incomplete, it is important to note that thiamine deficiency was produced by omitting or reducing the amount of this vitamin in the dietary supplements rather than by resorting to a destructive pro-

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cedure which could possibly also destroy other factors than thiamine.

A detailed description of the effects of thiamine deficiency on the state of health, the urinary thiamine excretion and the blood pyruvic acid in young pigs has been given elsewhere.[6] We wish to present here the details of the pathological changes found in the hearts of the experimental animals and to point out again our failure to detect any pathological changes in the nervous system.

Material and Methods

The present study deals with observations on two groups of pigs given crystalline vitamins and one group fed desiccated whole liver instead of these vitamins. The first group (1, "thiamine omitted"), were given riboflavin, 0.12 mg.; nicotinic acid, 1.20 mg.; pyridoxine hydrochloride, 0.20 mg.; choline chloride, 10.0 mg., and calcium pantothenate, 0.50 mg., per Kg. of body weight per day. The second group (2, "low thiamine"), were given 10 μg. thiamine hydrochloride per Kg. of body weight per day in addition to the above crystalline vitamins. The third group received none of these vitamins but were fed 1.5 gm. of desiccated whole liver per Kg. of body weight per day. This quantity contains about 40 μg., or less, of thiamine but is rich in all the other components of the B group.

Full details of the experimental procedure are found elsewhere.[6-9]

When the animals died or were killed, complete autopsies were performed. Blocks of all the tissues were fixed in formaldehyde and in one animal from each group all the tissues were imbedded in paraffin and stained with hematoxylin and eosin. In addition, the heart and lungs were carefully examined in all the animals, as were also the brachial, sciatic and vagus nerves, the sensory and motor nerve roots, the dorsal root ganglia and the spinal cord. The brains of three representative animals were also studied.

For comparison with these 9 animals we have examined sections of the myocardium of 45 pigs, some of which had received all the crystalline vitamins of the "B" group[7] while others had been deficient in one or more components of this complex.[7, 9] A number of these animals failed to grow normally and two were pigs whose food had been purposely restricted to study the effect of chronic inanition on the tissues.[8]

Observations

Group 1

Pig no. 6–53. This animal died after an experimental period of 156 days. During this time three episodes of vomiting, anorexia and loss of weight had occurred; accompanying these symptoms there was an elevation of the pyruvic acid level in the blood. The first two episodes

were relieved by the administration of thiamine and the blood pyruvic acid decreased to normal values. During the last 3 weeks of life, which was maintained by giving small and inadequate amounts of thiamine, the pyruvic acid level of the blood was constantly elevated. Vomiting and anorexia developed in the last week of life. On the day of death the animal was observed to become weak and short of breath. It walked reluctantly and unsteadily. The heart sounds were of good quality. Cyanosis developed suddenly and was soon followed by loss of consciousness and death.

The carcass weighed 63.8 Kg.; the heart, 280 gm. or 0.44 per cent of the body weight. The heart was dilated and the musculature was pale and flabby. In the myocardium of the left ventricle there were numerous fine yellow streaks several millimeters in greatest diameter. The valves were normal. The lungs were hemorrhagic and edematous. The other organs were not remarkable.

Microscopically, there were widespread lesions in the auricles and ventricles. These consisted of areas of necrosis of muscle fibers. Such foci were infiltrated by polymorphonuclear neutrophils, a few eosinophils and monocytes. In the older lesions there were connective tissue cells as well. The initial change seemed to be a loss of striation accompanied by vacuolization and hyalinization of the fiber; leukocytes then appeared. The lesions varied from small foci (Fig. 3) involving only a few fibers to large areas covering several low-power microscopical fields (Fig. 2). The latter were undoubtedly the areas seen grossly. In older lesions no intact muscle fibers could be found; here the predominant cells were mononuclear, consisting of macrophages and connective tissue cells. Scattered among these in much smaller numbers were neutrophils and eosinophils. No large necrotic areas were encountered in the auricles, where the lesions consisted of minute focal necroses. The latter were widespread. In some of the intact, normal appearing fibers vacuoles could be seen.

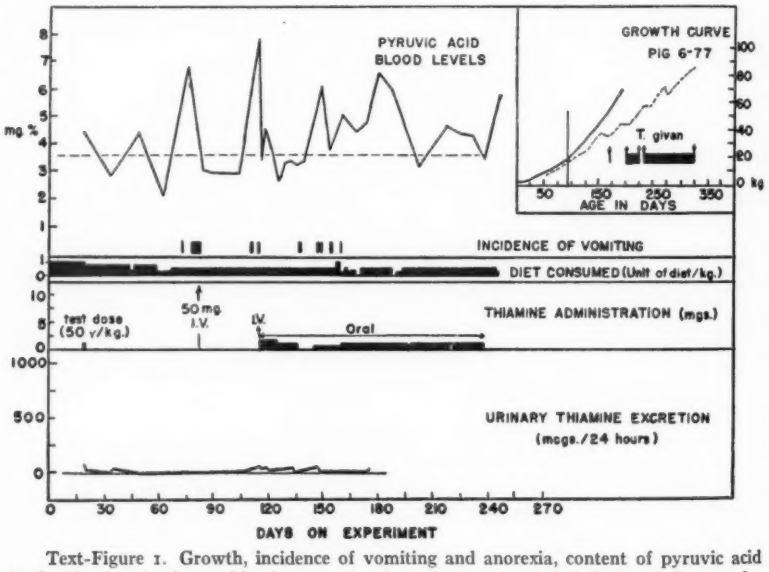
In the pulmonary alveoli, red blood cells and fluid could be found. The liver showed no chronic passive congestion. Histological examination of the nervous tissues revealed no lesions.

Pig no. 6–67. This animal lived 83 days on the diet free of thiamine. No ill effects were observed until the deficiency had lasted 74 days, when the animal began to vomit. On the 75th experimental day the content of pyruvic acid in the blood was found to be very high (6.8 mg.), and 8 days later the animal was found dead. It is of interest to point out that the pyruvic acid level of the blood had been somewhat elevated (4.9 and 4.5 mg.) or at the upper limits of normal (3.5 mg.) for the last 47 days and had been very high for about 1 week; thiamine was administered on only one occasion, 64 days before death.

The heart was not weighed. Microscopically, only a section of the left ventricle was available for study. Here were areas in which there was diffuse leukocytic infiltration between the muscle bundles; some of the latter were necrotic. In contrast to pig no. 6–53, there were no large localized lesions.

The lungs were edematous. The other tissues were autolyzed. Study of the nervous tissues revealed no degeneration.

Pig no. 6–77. This animal died after an experimental period of 246 days. During the midportion of this period there were numerous episodes of vomiting and anorexia, accompanied by elevations of pyruvic acid in the blood (Text-Fig. 1). Thiamine was administered from time to time to decrease the severity of the deficiency. The pyruvic acid content of the blood had remained elevated most of the time for the last 100 days. During the final week no thiamine was given and the pyruvic acid in the blood increased to 5.7 mg. At this time the animal appeared listless and was reluctant to move about.



Text-Figure 1. Growth, incidence of vomiting and anorexia, content of pyruvic acid in the blood and urinary thiamine excretion in a pig (no. 6–77) fed the basal diet supplemented only with riboflavin, nicotinic acid, pyridoxine, choline and pantothenic acid. Thiamine was given only as a "test dose," as a temporarily curative dose of 50 mg., and later only in subminimal doses (20 to 30 μg. per Kg. of body weight daily). The latter were furnished in order to produce a chronic deficiency of thiamine.

The interrupted line in the section representing pyruvic acid indicates the upper limit of pyruvic acid in animals not deficient in thiamine.[6]

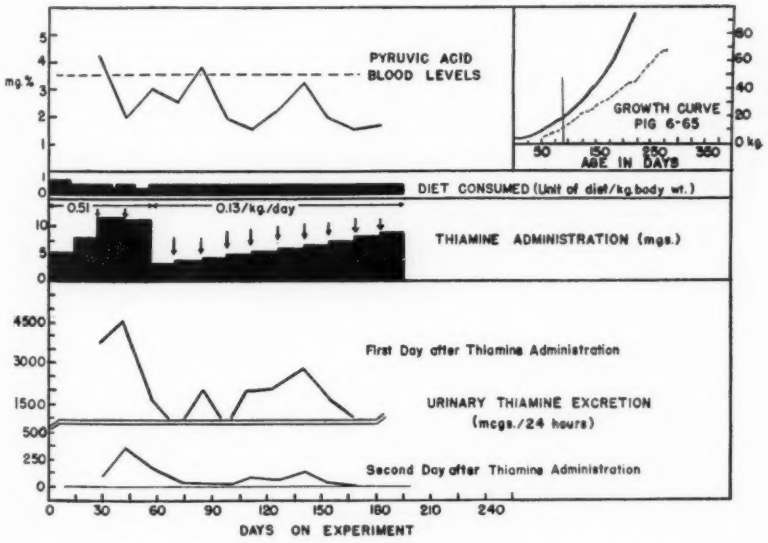
The continuous line in the growth curve represents the growth of pigs given a mixed diet at the Beltsville Research Center and fed and handled according to record of performance procedure. The interrupted line indicates the growth of the animal studied.

As it was exercised, marked dyspnea and pronounced cyanosis developed. With deepening of cyanosis the animal collapsed and, after renewed activity, passed into coma following a series of convulsive movements. Death occurred about 1 hour later.

The carcass weighed 84 Kg. The heart, which was dilated, weighed 500 gm. (0.60 per cent of the body weight).

Microscopically there were numerous lesions scattered throughout both auricles and ventricles. They were more extensive in the right auricle than in the left. In the ventricles there were focal areas of necrosis, some of which covered a quarter of a microscopical field. There were fresh, smaller foci as well. In addition to the areas of necrosis associated with leukocytic infiltration, there were many places where the muscle fibers had disappeared and connective tissue had taken their place. Such foci were interpreted as being areas which had formerly been necrotic and were now healed.

The lungs showed edema. The hepatic sinuses were engorged with blood but there was no atrophy of the central liver cells. Examination of the nervous tissue, including the brain, revealed no abnormalities.



Text-Figure 2. Growth, diet consumption, content of pyruvic acid in the blood and urinary thiamine excretion in an animal given thiamine in adequate amounts in addition to riboflavin, nicotinic acid, pyridoxine, choline and pantothenic acid. For comparison with Text-Figure 1. Legends as in Text-Figure 1.

Thiamine was given every other day. Under urinary thiamine excretion, "first day" refers to the 24-hour period following thiamine administration; "second day" to the succeeding 24-hour period.

Group 2

Pig no. 6–91. This animal was found dead after 37 days on the "thiamine-low" regimen. It had been entirely well and had gained steadily during the experimental period. No studies were made of the pyruvic acid content of the blood.

The heart was dilated and weighed 95 gm. The carcass weighed 20.6 Kg., so that the heart comprised 0.45 per cent of the body weight.

On microscopical study, lesions were found in both auricles but none was present in either ventricle. The right auricle (Fig. 1) was more severely involved than the left. There were numerous scattered necrotic muscle fibers about which leukocytes were gathered. The whole gave the picture of a severe diffuse myocarditis. In the lungs there was fluid in some of the alveoli. The liver was normal. Sections of the nervous tissues, including the brain, showed no lesions.

Pig no. 6–90. This animal died suddenly after receiving the "thiamine-low" diet for 57 days. During the last 10 days of life it vomited several times and began to lose weight. No studies of the pyruvic acid content of the blood were made.

The pig weighed 20.6 Kg. and the heart, which was dilated, weighed 140 gm., being thus 0.67 per cent of the body weight.

Microscopically, there were lesions in both auricles and ventricles. The left auricle was more extensively involved than the right; the same was true of the ventricles. There were focal and confluent diffuse areas of necrosis with cellular infiltration; the latter type of lesion was more prominent, however, especially in the ventricles. The interventricular septum was more severely damaged than the walls of the ventricles. Fat stains revealed foci of fatty infiltration of the muscle fibers in the fresh lesions. The lungs showed edema. The nervous tissues were normal.

Pig no. 6–92. After having received the "thiamine-low" diet for 141 days, this animal was sacrificed. Between the 41st and 91st days of the experiment signs of deficiency, in the form of vomiting and anorexia and elevated pyruvic acid content of the blood, were present. Because of the deaths of the other two animals in this group (nos. 6–90 and 6–91), this pig was given more thiamine than the others. As a result, with the exception of elevated pyruvic acid on a single occasion on the 123rd day of the experiment, no signs of deficiency whatever were detected in this animal during the last 41 days of its life. The animal was killed because of a spreading infection of one hind leg.

The carcass weighed 39.2 Kg. and the heart, 220 gm., being 0.56 per cent of the body weight. Grossly the heart was normal.

On microscopical examination the heart was also entirely normal. There were widespread abscesses in the lungs. The nervous tissues showed no lesions.

Group 3

Pig no. 6–54. This animal died suddenly after having received the desiccated liver supplement for 320 days. For the first 120 days it seemed well. During the final 200 days there were at least six episodes of vomiting, accompanied by loss of weight and elevation of the blood pyruvic acid and interrupted on several occasions by the administration of thiamine. The concentration of pyruvic acid was elevated above normal almost continuously for the last 9 weeks of life.

At autopsy the animal weighed 46 Kg. The heart was tremendously dilated and weighed 350 gm. This represented 0.76 per cent of the body weight.

Microscopically there were widespread lesions of the muscle fibers in both auricles and ventricles, particularly striking in the former. The interstitial tissue was diffusely infiltrated with polymorphonuclear and mononuclear leukocytes. Many muscle fibers were necrotic. In the ventricles there were a few confluent focal areas, but for the most part the change here was diffuse, too. There were numerous foci where the muscle fibers had been replaced by collagenous tissue (Fig. 4). The pulmonary alveoli contained fluid. The liver was normal in appearance. Sections of the nervous tissues, including the brain, were entirely normal.

Pig no. 6–62. This animal was sacrificed after receiving desiccated liver for 239 days. During the midportion of this period vomiting and loss of weight occurred and the blood pyruvic acid was observed to be elevated. The pyruvic acid at first dropped spontaneously to normal, a change which was interpreted as being due to decreased food consumption. Soon the pyruvic acid level rose again, however, and this was accompanied by vomiting and anorexia. Following the daily administration of thiamine the pyruvic acid in the blood fell and remained normal for the last 45 days of life, when the animal was sacrificed.

At autopsy this pig weighed 74 Kg. The heart appeared normal, weighing 270 gm. or 0.36 per cent of the total body weight.

Microscopically no lesions were seen in the auricles or ventricles. The nervous tissue was normal.

Pig no. 6–79. This animal was sacrificed after it had received desiccated liver for 230 days. On the 120th day, although there were no clinical signs, the pyruvic acid level of the blood was found to be elevated. With some decrease in food consumption it dropped, but

later rose again; the animal began to vomit coincidentally with this rise. The pyruvic acid concentration remained elevated or at the upper limit of normal until 30 days before death when thiamine was administered. From this time until death the pyruvic acid content of the blood was normal.

At autopsy the carcass weighed 76.4 Kg. The heart, which was not dilated, weighed 330 gm., being 0.43 per cent of the body weight.

Microscopically the myocardium was entirely normal. The nervous tissue showed no lesions.

Controls

In none of the 45 control animals, all of which received thiamine, were any changes found in the myocardium. Some of these pigs were lacking in vitamins of the B group other than thiamine and grew very poorly, and two were given reduced amounts of food adequately supplemented with vitamins. Thus it is evident that partial inanition is not a cause of the lesions observed.

Discussion

We have observed lesions in the auricular and ventricular musculature of six out of nine pigs receiving a diet adequate in all known respects except for its thiamine content. Five of these six animals received crystalline vitamins other than thiamine as the source of the vitamin B complex, while one was fed desiccated liver known to be poor in thiamine but probably adequate otherwise as a source of the B complex. The lesions consisted of focal or diffuse necrosis of myocardial fibers. Such areas were infiltrated by leukocytes and, in the more chronic cases, were replaced by connective tissue cells so that scars resulted.

In the two animals whose mode of death was observed (nos. 6–53 and 6–77) the clinical picture was that of heart failure with labored breathing and cyanosis, which were made worse by exercise. The four other animals showing lesions in the myocardium died quite unexpectedly during the night. The three pigs failing to show any myocardial lesions had been sacrificed. These animals appeared well at the time they were sacrificed and had been receiving thiamine in doses sufficient to reduce the pyruvic acid of the blood to normal levels for the preceding 30 to 45 days (Table I).

In the animal (no. 6–91) dying very early, after deprivation of thiamine for only 37 days, lesions were found in the auricles but none could be discovered in the ventricles, even after numerous sections were made. In the animals dying after longer periods of deficiency,

TABLE I

Relation of Terminal State of Thiamine-Deficient Animals to Pathological Findings

Experimental group	Pig no.	Duration of experimental deficiency	Time required for onset of first symptoms	Terminal state		Focal necrosis		Lungs
				General condition	Blood pyruvic acid	Auricles	Ventricles	
		(*days*)	(*days*)		(*mg./100 cc.*)			
Group 1, "thiamine omitted"	6–53	156	72	Cardiac failure	7.4	+	+	Congestion and edema
	6–67	83	74	Sudden death	6.8	?	++	Edema
	6–77	246	74	Death following exercise	5.6	+	++	Edema
Group 2, "low thiamine"	6–90	57	41	Sudden death		++	+	Edema
	6–91	37	..	Sudden death		++	o	Edema
	6–92	141	41	Good; sacrificed	3.1	o	o	(Abscesses)
Group 3, desiccated liver	6–54	320	117	Sudden death	7.3	+	+	Edema
	6–62	239	115	Good; sacrificed	3.2	o	o	o
	6–79	230	135	Good; sacrificed	2.5	o	o	o

lesions were observed in the ventricles as well as in the auricles. In no instances were pathological changes found in the ventricles when they were lacking in the auricles. This suggests that the auricles may be more sensitive to thiamine deficiency than the ventricles. It is of interest in this respect that Muus, Weiss and Hastings,[10] using tissue from thiamine-deficient rats, noted that there was a reduction from normal in the O_2 consumption of the auricular muscle *in vitro* while that of the ventricles remained unchanged. They were unable, however, to find any correlation between the degree of reduction of O_2 consumption of the auricle and the severity of the deficiency.

In two animals in which episodes of thiamine deficiency, marked by anorexia, vomiting and elevation of pyruvic acid in the blood, had been observed at intervals during their course, scars indicative of former necrosis of the myocardium were found. It must be pointed out, however, that in the three pigs sacrificed at a time when they showed no signs of thiamine deficiency, no fresh lesions were observed in the myocardium

and no scars could be found as evidence of earlier episodes of deficiency such as these pigs had suffered. Connective tissue stains were made in addition to hematoxylin and eosin preparations but no indication of scarring could be made out. It would appear, then, that while scars marking the sites of previous necrotic myocardial fibers may be found in some animals, the lack of such scars does not necessarily indicate that thiamine deficiency has not been present.

A constant finding in the pigs showing lesions at autopsy was marked cardiac dilatation. When the size of the hearts of the experimental group was compared with that of controls it was also found that some of the former were enlarged in respect to the body weight. From observations on normally growing animals receiving all known vitamins we have found that the heart represents about 0.30 to 0.40 per cent of the total body weight. In four of the experimental animals the heart weight exceeded this and was as great as 0.76 per cent in one animal. It cannot be concluded, however, that these hearts were hypertrophied. When body growth is impaired, as was the case with the thiamine-deficient pigs, an alteration in the ratio of heart to total body weight occurs. Thus we have observed a heart weight-body weight ratio as high as 1.0 per cent in an animal receiving thiamine as well as riboflavin and nicotinic acid but growing very poorly as the result of deficiency of pantothenic acid and pyridoxine. Loss of body weight without corresponding loss of heart weight may give a false impression of cardiac hypertrophy.

In only a few of the many experiments on vitamin B_1 deficiency reported hitherto have specific lesions of the myocardium, such as those we have noted, been described and in none have they been as extensive as those we have observed. Swank,[1] using a diet partially deficient in thiamine, found myocardial lesions in pigeons that had exhibited gross evidence of cardiac failure. These consisted of scattered foci of necrotic muscle fibers which in two instances were infiltrated with leukocytes. Observations on thiamine-deficient dogs by Swank, Porter and Yeomans [2] revealed small scattered foci of necrotic muscle fibers in 3 out of 14 instances. Many of these foci were infiltrated with polymorphonuclear leukocytes. One animal showed a "loose scarring of the myocardium." In 7 pigs, on a diet supplemented with sulfite-treated liver and whey, Van Etten, Ellis and Madsen [3] found scattered areas of "atrophy and necrosis" of heart muscle fibers which were considered suggestive of infarction. In foxes dying of Chastek paralysis, a disorder which has been attributed to thiamine deficiency, Evans, Carlson and Green [11] have described areas of necrosis of myocardial fibers and, in a few instances, proliferation of connective tissue.

In human beriberi, pathological observations have for the most part been limited to gross study of the heart; few detailed microscopical studies have been made. There is controversy whether the chambers of the right side are both hypertrophied and dilated or merely dilated. Vedder [12] stated that "the heart is always considerably enlarged, particularly on the right side." In the majority of the cases studied by Weiss and Wilkins [4] "the weight of the heart was normal and there was moderate dilatation of the right ventricle." From an analysis of the literature one is struck by the paucity of actual measurements of the heart weight, reliance having been placed on its appearance. Studies of the weights of the individual ventricles are certainly much needed.

It does not appear that sufficient attention has been paid to the histological appearance of the "beriberi heart" to warrant any specific conclusions. Although Wenckebach [13] and Weiss and Wilkins [4] have noted "hydropic" degeneration of the myocardial fibers, the latter observers found the same change in other types of heart disease as well. It would be of particular interest in the light of our observations on pigs to study carefully the auricular musculature of patients dying of beriberi.

It should be pointed out that there are a number of reports of "isolated myocarditis" or "Fiedler's myocarditis," all of unknown etiology, which bear a striking similarity to the changes we have encountered in pigs. The possibility that some of these may have been instances of thiamine deficiency warrants consideration.

Cardiac lesions similar to those we have described have been noted in other experimental nutritional deficiencies. Focal necroses of myocardial fibers have been found in ascorbic acid deficiency in the guinea pig.[14] We have recently encountered similar lesions in rats whose dietary potassium had been greatly restricted.[15] In the latter animals, however, the lesions were much more prominent in the ventricles than in the auricles; the rats, furthermore, did not die of cardiac failure. From the standpoint of potassium content the diet employed in the present studies was entirely adequate.

At one time it was suggested that the cardiac manifestations of human beriberi are due to degeneration of the vagus nerves or to respiratory paralysis.[16] These hypotheses are now discredited and our own observations give them no support; the vagus nerves were examined in all the pigs and were found to be normal. As to the "water retention" theory proposed by Wenckebach,[13] we have already pointed out that hydropic degeneration is found in hearts failing as the result of a variety of causes. It may be added that Wilkins and Cullen [17] found an increase in the water content of the myocardium of both

ventricles in the hearts of patients dying of congestive failure of various types.

Although it is evident that careful histological studies of the hearts of patients dying of beriberi are needed, it is to be expected that in some instances, at least, no lesions will be found. Judging by what is known of the function of thiamine, it is to be expected that a metabolic disorder of the myocardium occurs before anatomical changes take place and that death may ensue before recognizable lesions have developed.

It is of some interest to speculate as to the factors which may have led to the death of our thiamine-deficient animals and to consider the possible reasons for the development of the lesions in the myocardium. The age at which animals are placed on the deficient diet probably influences the interval of time in which the effects of the deficiency make their appearance, since the more rapidly growth is taking place the more thiamine may be needed. The early death of pigs nos. 6–91 and 6–90 is consistent with this view. Another factor, no doubt, is the degree of activity of the animals; a young, lively animal possibly being more likely to develop sudden failure through activity than a less energetic, older pig. In this connection it is of interest that Keefer [18] observed that cardiac failure in cases of beriberi occurred particularly in patients who showed little or no neurological disturbances and were therefore more active, and that Swank, Porter and Yeomans [2] noted the absence of cardiac failure in dogs which became paralyzed. The amount of food consumed is probably another important factor because with a reduced food intake the requirements for thiamine are lowered. We were able to observe in several animals that the pyruvic acid content of the blood fell when the animals developed anorexia and so consumed less food.

It has been suggested from time to time that pyruvic acid may be toxic and that the accumulation of this substance in the tissues may damage them. Haynes and Weiss [19] administered large amounts of pyruvic acid, sodium pyruvate and related substances to normal and thiamine-deficient rats. Although slight changes in heart rate and in the T waves were noted, they concluded that it is unlikely that the accumulation of metabolites is the important factor in the production of abnormal cardiac manifestations in deficient rats since the amounts necessary to produce changes were very great. Lu [20] came to the same conclusions. If pyruvic acid is itself toxic, one wonders whether it is necessary for the pyruvic acid to reach certain levels in the blood and to be maintained at such levels for some time before cardiac lesions will develop. In the experimental studies of Haynes and Weiss and of

Lu no sustained elevation of the blood pyruvic acid was produced. Furthermore, histological studies of the myocardium were not made. Our own data are insufficient to answer these questions.

In view of the rather general conception that thiamine is the "antineuritic vitamin," it is worth commenting on the absence of neurological changes, both clinical and pathological, in the pigs we have studied. Recent observations from this laboratory have shown that ataxia, accompanied by sensory neuron degeneration, may be produced in swine when either pantothenic acid or pyridoxine is omitted from the diet.[7] Up to the present time no investigations have been carried out in other animals fed thiamine-deficient diets supplemented with adequate amounts of the other crystalline vitamins of the B group. The experiments of Swank [1] in pigeons are invalidated because in the main no vitamins of the B group other than thiamine were administered and in other experiments autoclaved diets were fed. It is possible that by autoclaving, other vitamins than thiamine may be damaged.

The negative findings in pigs, together with evidence from the critical analysis of Meiklejohn[21] concerning the lack of proof of any relationship of thiamine to human polyneuritis, raise doubt in our minds as to the rôle of thiamine deficiency in the production of pathological changes in the nervous system.[22] It is quite possible that degenerative changes heretofore attributed to lack of vitamin B_1 may have been due to the lack of other vitamins removed from the diet by autoclaving, or missing as the result of other causes. In the light of the newer information concerning the number of vitamins composing the B complex and their distribution in foods, it can no longer be assumed that the human disorder, beriberi, is caused by the lack of one vitamin only. If we may judge by our studies in pigs,[6, 7] beriberi develops as the result of deficiency of two and probably three vitamins and no doubt other factors and deficiencies play a part as well.

Lesions similar to those observed in Wernicke's disease have been described in pigeons by Alexander, Pijoan and Myerson [23] and in foxes suffering from Chastek paralysis by Evans, Carlson and Green.[11] Both groups of investigators found that the administration of thiamine prevented the development of the lesions. It should be pointed out, however, that a basal diet of polished rice supplemented only with riboflavin and vitamins A, C and D was employed by Alexander, Pijoan and Myerson. The presence of a "severe degree of lipoidosis" in the liver of the foxes studied by Evans, Carlson and Green makes one wonder whether in these experiments also other dietary factors were lacking as well as thiamine. In the present study examination of the brains of three representative animals failed to reveal any lesions.

Summary

1. Cardiac dilatation without hypertrophy, and focal and diffuse myocardial necrosis were the characteristic findings in six pigs dying of thiamine deficiency. In one instance the areas of necrosis were so large that they could be seen with the naked eye. The lesions were infiltrated with polymorphonuclear and mononuclear cells.

2. Areas of necrosis were found only in the auricles in the pig dying after the shortest period of deficiency. In the remaining animals lesions were found both in the auricles and in the ventricles.

3. In two animals which had passed through several episodes of severe thiamine deficiency, scars marking healed necrotic lesions were found.

4. In three experimental animals that were sacrificed at a time when no clinical or chemical evidence of thiamine deficiency was present, no pathological changes were found.

5. No cardiac lesions were observed in a large number of animals dying as the result of other types of vitamin deficiency or in those in which inanition alone was produced.

6. No changes whatever were found in the nervous systems of the thiamine-deficient pigs.

Vitamins used in these experiments were furnished by Merck and Company, Inc., and yeast by Mead Johnson and Company. Dr. Theodore L. Danforth, Jr., and Dr. Raul Alcayaga assisted in some of these studies and technical aid was given by Adolph Suksta, Lottie Lowenstein, Eleanor Collins, Eva Hodgens, Elaine Cohen, Gertrude Merr and William Mock. Photomicrographs were made by Milton Kougl.

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[*Illustrations follow*]

DESCRIPTION OF PLATE

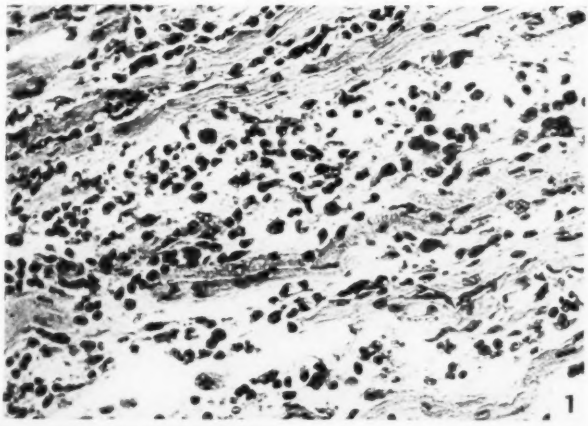
PLATE 40

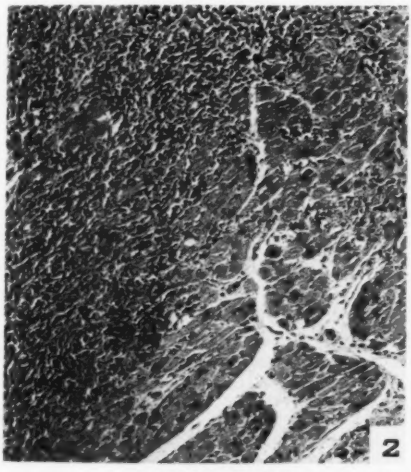
FIG. 1. Photomicrograph of right auricle of pig no. 6–91, dying after 37 days on the "thiamine-low" regimen. There is extensive leukocytic infiltration where myocardial fibers have become necrotic. × 200.

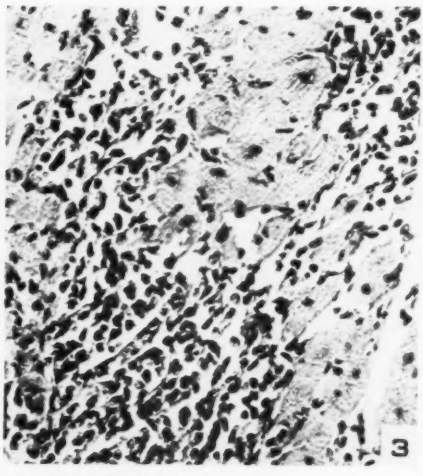
FIG. 2. Photomicrograph of left ventricle of pig no. 6–53 to show edge of large, macroscopical lesion. × 90.

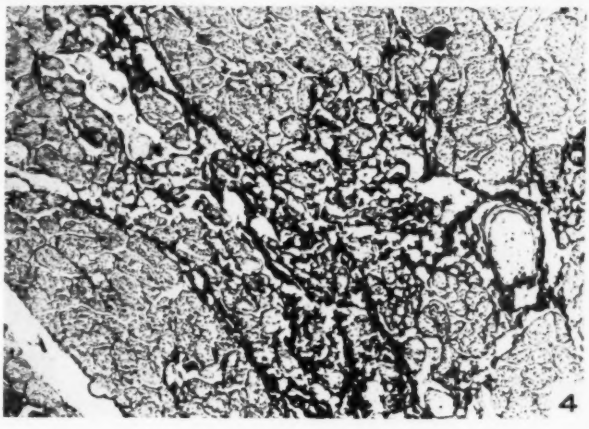
FIG. 3. Photomicrograph of left ventricle of pig no. 6–53 showing foci of leukocytes, mainly mononuclear, in areas where muscle fibers have disappeared. × 200.

FIG. 4. Photomicrograph of left ventricle of pig no. 6–54 to show an area where the myocardial fibers have disappeared and have been replaced by collagenous fibers. This is far in excess of the normal amount of connective tissue. Mallory's aniline blue stain. × 200.









NEOPLASTIC DISEASE OF THE PANCREAS OF SNAKES (SERPENTES)*

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Some years ago, in the course of a routine autopsy of a pine snake (*Pituophis sayi*), the pancreas attracted attention because of its apparent enlargement. Upon histologic examination, the parenchyma was found to have been largely replaced by tissue which appeared to be carcinoma.[1] Since neoplasms of any sort have rarely been found in reptiles,[2] this observation led to microscopic examination of the pancreas of each animal of all species of this class of vertebrates that died while on exhibition in the Zoological Garden. As a result, changes which corresponded closely to epithelial neoplasia were found to be comparatively common in the pancreas in several species of snakes, but not in other types of reptiles. Of a series of 73 animals, representing three families of the order Serpentes, 24 had developed lesions of this character, and in 9 of these, the greater part of the gland was replaced by carcinoma.[3] Changes in others of this group could be interpreted as developmental stages of the disease, but either were too far advanced to permit an opinion of their origin and development or they were confused by diffuse inflammatory reactions, abscesses and autolysis. Hence, it seemed essential to further study to obtain animals demonstrating earlier and uncomplicated stages of the process.

Members of each of the families that were included in this series had developed this disease, but the majority of the group, and of the animals in which the pancreas was involved, were species of Crotalidae and Colubridae native to North America. The greater number of these had died within 4 or 5 months after capture. It was assumed, therefore, that if numbers of the more susceptible species were killed after 2 or 3 months of captivity, they would provide many examples of developmental stages of the disease.

Choice of species for purchase and slaughter was determined by cost, space requirements and ability of collectors to supply them in quantity, as well as by apparent susceptibility. The ones selected were the prairie rattlesnake *(Crotalus confluentus)*, the Jersey pine snake *(Pituophis melanoleucus)* and the black racer *(Coluber constrictor)*. These animals were kept in exhibition cages at the Zoo-

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logical Garden until killed. It developed also that the pancreases of the diamond-backed rattlesnakes *(Crotalus adamanteus)*, and of water moccasins *(Agkistrodon piscivorus)*, apparently the most and the least susceptible of the Crotalidae, might be obtained in fixative from a Florida canning plant which prepares snake meat commercially. These snakes were said to have been killed within a few days after capture.

While these animals were being assembled and studied, microscopic examinations of all other specimens that became available through routine autopsies were continued. In no instance was any change which might be interpreted as a stage of the disease found in the pancreas of reptiles other than snakes. And, contrary to expectations, it was soon evident that the character and extent of lesions to be found in sections were not predictable from gross examination, nor did the frequency of the disease in snakes killed for examination approximate that in those dying spontaneously. Hence, neither biopsy nor transplantation was attempted. Efforts to trace the development of the disease and to determine its nature have been limited, therefore, to interpretation of a variety of changes found upon microscopic study of the pancreas.

The Normal Pancreas

The pancreas in the several species of snakes included in the present series is a solid body, oval, reniform or angular in outline, enclosed by the mesentery and closely applied to the serosa of the duodenum a few centimeters below the pylorus. Its transverse diameter approximates that of the gut and its length is about twice its width. The common bile ducts (these usually are multiple but within a common sheath) pass through its midportion and enter the intestine close to the pancreatic ducts. In color the organ may vary from dull white to pale pink or brown, and, in consistency, it suggests a lymph node. The spleen, which usually is much smaller than the pancreas, lies on or near the upper pole, or the upper pole may be drawn out into an elongated, narrow structure, as it is in birds, to unite with the spleen, a feature more characteristic of the Boidae, than of other types of snakes in this series. In any event, the spleen and pancreas often are incompletely separated by distinct capsules, and tissues may be mixed about the line of union, a feature which probably explains the occasional presence of neoplastic epithelium within the spleens of certain of the animals of this series.

In the normal pancreas of snakes, cells of the duct system and acini are closely similar to those of mammals and birds but, as a rule, the parenchyma is not subdivided (Fig. 1). Stroma is scanty except at

the periphery and about the larger ducts. Islands of Langerhans usually are composed of eosinophilic, columnar cells, or these cells may contain eosin-staining granules. These bodies commonly are large and irregular. They are found chiefly in the region adjacent to the spleen and occasionally one or more may be seen within the splenic tissue.

Since most of the glands examined were less than 2 cm. in length, complete sections through the long axis, and in a plane parallel with the course of the larger ducts, were easily prepared. These gave maximum areas for study and usually included views of the spleen and of several large pancreatic ducts.

Neoplastic Disease of the Pancreas

Changes that have been interpreted as initial phases in the development of epithelial neoplasia were found in four of the snakes that died in captivity, as well as in three *Crotalus confluentus* and three *C. adamanteus* which were killed for examination. The pancreases of these animals contained small scattered foci in which acini were distorted and dilated, and epithelium within them was detached and fragmented or replaced by flattened, indistinctly outlined, pale-staining cells, the nuclei of which were pleomorphic, hyperchromatic and irregularly distributed (Figs. 2 and 3). Of these lesions, those in which epithelial degeneration was the pronounced feature were not well outlined, but within them acini were clearly separated from one another and from surrounding glandular elements as if by local edema (Fig. 2). When glandular epithelium had been replaced by other cells, the focus contained fewer subdivisions, fibrous tissue cells were increased and polymorphonuclear leukocytes and lymphocytelike cells accumulated in small numbers within the involved area and in the surrounding tissues. Often fibrous tissue seemed to outline the area rather sharply.

In only one case did these early focal lesions center about obviously necrotic tissues, and this in an animal dying of acute bacterial infection with many small thrombi in various organs. Thrombi accounted for focal necrosis of the pancreas in this instance. Search for the primary causes of the focal lesions was fruitless in all other animals, however.

Glandular tissue of the pancreases which contained the small focal lesions usually was otherwise unchanged. Normal acini lay close by the degenerating ones. But in these and in all other specimens in which were found lesions that have been interpreted as some stage in the development of epithelial neoplasia, cells of the duct system presented various grades of hyperplasia, usually moderate, sometimes marked, but the various grades were not associated closely with the degree of change in the acini.

Larger focal lesions which could have developed from the smaller, more isolated ones were found more frequently and were associated with more extensive changes of the gland. These occurred in 15 snakes that died spontaneously, as well as in four *Coluber constrictor* that were killed for examination. In addition to increased size of these second stage lesions, abnormality of epithelium within them was more pronounced than in the smaller ones, stroma was increased in and about them and some of them, especially those which formed at the periphery of the pancreas, had the appearance of expanding masses (Figs. 4 and 5). Rather than retaining some suggestion of acinous pattern, epithelial cells were elongated, flattened, indistinctly outlined and arranged into small branching cords and ducts. These lay in loosely arranged masses with scanty fibrous tissue cells between, but fibrous tissue increase was more pronounced about the periphery of lesions, in the remnants of normal acinar tissue, and in the capsule rather than within the lesions themselves. In some instances (Fig. 4), polymorphonuclear leukocytes and large and small lymphoid cells were numerous in and about the lesions, but, as in the examples of earlier stages of the disease, abscesses were not formed.

Glandular tissues about these larger focal lesions usually presented well defined changes. Over rather wide areas acini were dilated and their lumina filled by eosin-staining coagulum. Appearances of glandular epithelium within these areas varied; some acini were well preserved but many were undergoing degeneration. However, there was nothing to indicate that these larger foci had expanded by transformation of the altered units into the ducts and cords mentioned earlier. Rather, they seemed to be degenerating as the new-formed structures enlarged. But in spite of these relatively extensive focal changes, considerable amounts of unaltered glandular tissues usually remained at this stage of the disease.

Many, if not all, of the examples of more advanced forms of this disease may be interpreted as having been formed by continued growth of the centers of abnormal regeneration in one or in several parts of the gland. In 16 animals, lesions were considerably more extensive than those of the second group but, still, islands of comparatively normal tissue were present. These cases have been classed as a third stage of the disease. All of these animals died while on exhibition. In them, epithelial elements of the focal lesions assumed a more definite organization, forming compact masses of irregular tubules, or apparently expanding adenomata (Figs. 6 and 7). Their component cells were clearly defined and pleomorphic, with dense cytoplasm and large, irregular, vesicular nuclei, and the cell pattern more closely resembled

that characteristic of carcinoma. These lesions seemed to be expanding, and compressing surrounding tissues rather than being separated from them by edema. Stroma within them was increased and compact. In the remainder of the gland, stroma was also increased and the fibers separated. Leukocytes and lymphoid cells likewise were present in increased numbers, and many large phagocytic cells (epithelioid cells) were noted.

Intervening glandular tissue retained a normal pattern fairly well, sometimes with considerable degeneration of the epithelium and dilatation of acini. As a rule, it amounted to less than one-half of the organ. In these cases, as well as in those exhibiting more advanced changes, islet tissue could not be identified.

Finally, a group of ten animals, all of which died in captivity, supplied the specimens in which lesions were of a type that suggested carcinoma most strongly. In so far as could be determined by sections through the long axis of the gland, neither acinar nor islet cells remained in recognizable form. Microscopic appearances of these specimens varied. Three were largely fibrous tissue with islands of epithelium forming adenomatoid structures. In the remainder, fibrous tissue was less abundant, and epithelial elements more closely placed but forming, nevertheless, equally abnormal structures (Figs. 8 and 9). These variations were no greater than might be expected in any group of neoplasms of glandular tissues. In three animals of this group the capsular tissues contained small adenomatous growths which suggested local extension, and, in two, similar structures were present in the wall of the gut adjacent to the pancreas, which probably did not indicate local invasion, but rather that the displaced tissue responded as a unit of the organ. Those specimens of this group which were largely scar tissue probably resulted either from local infection and abscess formation, or from unexplained degeneration of the abnormal glandular tissue. There was evidence that both of these processes were operating.

Species Involved and Frequency of Disease

Failure to list the species of snakes that died spontaneously and in which this disease of the pancreas occurred is intentional. There seemed to be no point in this, since no one variety predominated in any particular subgroup. Instead, the entire series has been tabulated to show the families in which the disease occurred and the number of specimens of each that were examined. Two of the families, Crotalidae and Colubridae, have been subdivided to genera and species because they contained the bulk of the series and among them there apparently

were differences in susceptibility. The pancreases of reptiles of many other varieties were examined, but only those families in which the disease occurred have been included in the following table.

Table I contains records of 397 specimens, of which 136 died while on exhibition in the Zoological Garden. These animals were members of five families of the order Serpentes: Crotalidae, Colubridae, Boidae, Elapidae and Viperidae. Column 1 of the table contains the number of animals in each of these groups, and the second column shows the

TABLE I

Number and Variety of Snakes Examined and Frequency in Each Group of Pancreatic Lesion of a Type that Suggested Epithelial Neoplasia

Families	Died		Killed	
	Number examined	Number positive	Number examined	Number positive
CROTALIDAE	92	33	184	6
Crotalus adamanteus	25	15	56	3
Crotalus confluentus	20	3	115	3
Crotalus horridus	17	6	0	0
Crotalus ruber	11	7	0	0
Sistrurus miliarius	5	1	0	0
Agkistrodon mokasen	4	1		
Agkistrodon piscivorus	10	0	13	0
COLUBRIDAE	30	6	77	4
Pituophis melanoleucus	7	1	24	0
Pituophis sayi	4	1	0	0
Coluber constrictor	3	2	53	4
Lampropeltis getulus	2	2	0	0
Natrix sipedon	14	0	0	0
BOIDAE	5	3		
ELAPIDAE	4	2		
VIPERIDAE	5	1		
Total	136	45	261	10

number of cases in which there was some grade of pancreatic disease which might be interpreted as part of the process of epithelial neoplasia. This part of the series supplied 45 of the positive cases, which is to be contrasted with 7 cases among 195 snakes that were killed for examination. It will be recalled that, of the 261 specimens listed in column 3 of the table, 56 *Crotalus adamanteus* and 13 *Agkistrodon piscivorus* were killed at the canning plant. Thus the 10 positive cases among snakes killed for examination contain 3 that were not subjected to the influence of captivity.

The number of representatives of any one of the species of Crotalidae and Colubridae listed in the table is rather too small for satisfactory comparison of incidence, but apparent differences in susceptibility may be noted. In particular, of 25 specimens of *Crotalus adamanteus*, 15 were involved, while none of 10 *Agkistrodon piscivorus*

developed lesions of the pancreas. Among the Colubridae, the contrast is not so sharp, but 6 of 16 snakes of four species developed the disease and none of 14 *Natrix sipedon* was involved. In general, failure to develop this condition has been paralleled by the ease with which animals of various species become adjusted to captivity.

Discussion

Obviously the title of this paper is not completely justified except by the 10 cases which have been designated as stage four in the development of this disease of the snake's pancreas. Whether or not these specimens may be regarded as true neoplasms seems to depend largely upon definition. Certainly their morphology appears to warrant this classification, although metastases were not found and local extension occurred infrequently. Perhaps the absence of metastases may be accounted for by the small number of cases in the series and does not reflect the growth characters of the abnormal tissues. Lucké examined a much larger series of carcinomata of the kidney of *Rana pipiens* before finding secondary growths [2] and observed that, in comparison with similar tumors of man, this neoplasm of frogs metastasized later in the course of its development.[4] Moreover, the lesions of snake's pancreas affect the whole organ, replacing acinar and islet tissues completely. Thus, possibly, interference with pancreatic function ends life before growth characters of the new tissue may be fully expressed.

Again there can be no certainty that the focal lesions are phases in the development of the more extensive changes; but if one bases judgment upon morphology only, this seems to be a reasonable assumption. Granting it to be true, then the various stages of the process are examples of regeneration and hyperplasia which occasionally undergo neoplastic change but which more frequently are found in intermediate states. For it seems clear that the initial stages of the disease are simply irregular focal degeneration of acinar epithelium followed by replacement of these cells by ones which resemble those of the terminal ducts, which is entirely in keeping with experimental studies of regeneration of pancreatic tissue of mammals.[5] But this disease of the snake's pancreas is unique, not so much because of its frequency, as because it emphasizes the possible difference in susceptibility to neoplasia inherent in the organs and tissues of various animal types.

Search for a cause or causes of the primary foci of degeneration has been fruitless. Blockage of ducts by inflammatory changes has not been definitely established in any of the animals. Acute inflammatory disease of the intestine which might produce such an effect often was

present in the animals that died spontaneously, but not always so. And this factor could be ruled out completely in the animals that were killed for examination. Parasitic organisms, especially species of Sporozoa, which might stimulate hyperplasia of ductal epithelium, were found only in a few snakes, none of which have been included in the present series since changes associated with these organisms were distinctly different.

Summary

The acinar tissue of the pancreas in many species of snakes undergoes unexplained focal necrosis followed by abortive regenerative growth, apparently of the terminal ducts, producing small, edematous adenomalike structures. These areas presumably enlarge, and with their enlargement, leukocytes infiltrate the organ, fibrous tissue is increased and there is further degeneration of acinar and islet epithelium, until occasionally the whole organ is replaced by tissue which has the histologic characters of carcinoma. In a series of 136 snakes of five families of the order Serpentes, all of which died in captivity, 45 presented some stage in the development of this disease. But of 261 snakes of species that seemed most susceptible to the disease, killed for examination 60 to 90 days after capture, only 10 had developed lesions of this sort and none of them presented the more advanced stages of the disease.

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[*Illustrations follow*]

DESCRIPTION OF PLATE

PLATE 41

FIG. 1. Normal pancreatic tissue from a black racer (*Coluber constrictor*), showing the arrangement of acini. × 90.

FIG. 2. A first stage lesion of the pancreas of a black racer (*Coluber constrictor*), dying 3 months after capture. This area shows unchanged acini about acini that are undergoing degeneration and being replaced by epithelium which probably grew in from the terminal ducts, local edema and accumulation of phagocytic cells. × 90.

FIG. 3. Central part of Figure 1. × 200.

FIG. 4. Second stage lesion of the pancreas of a diamond-backed rattlesnake (*Crotalus adamanteus*), dying after 40 days in captivity. This had formed adjacent to the spleen, and acinar tissue has been replaced by thin-walled tubules and loosely arranged connective tissue. × 90.

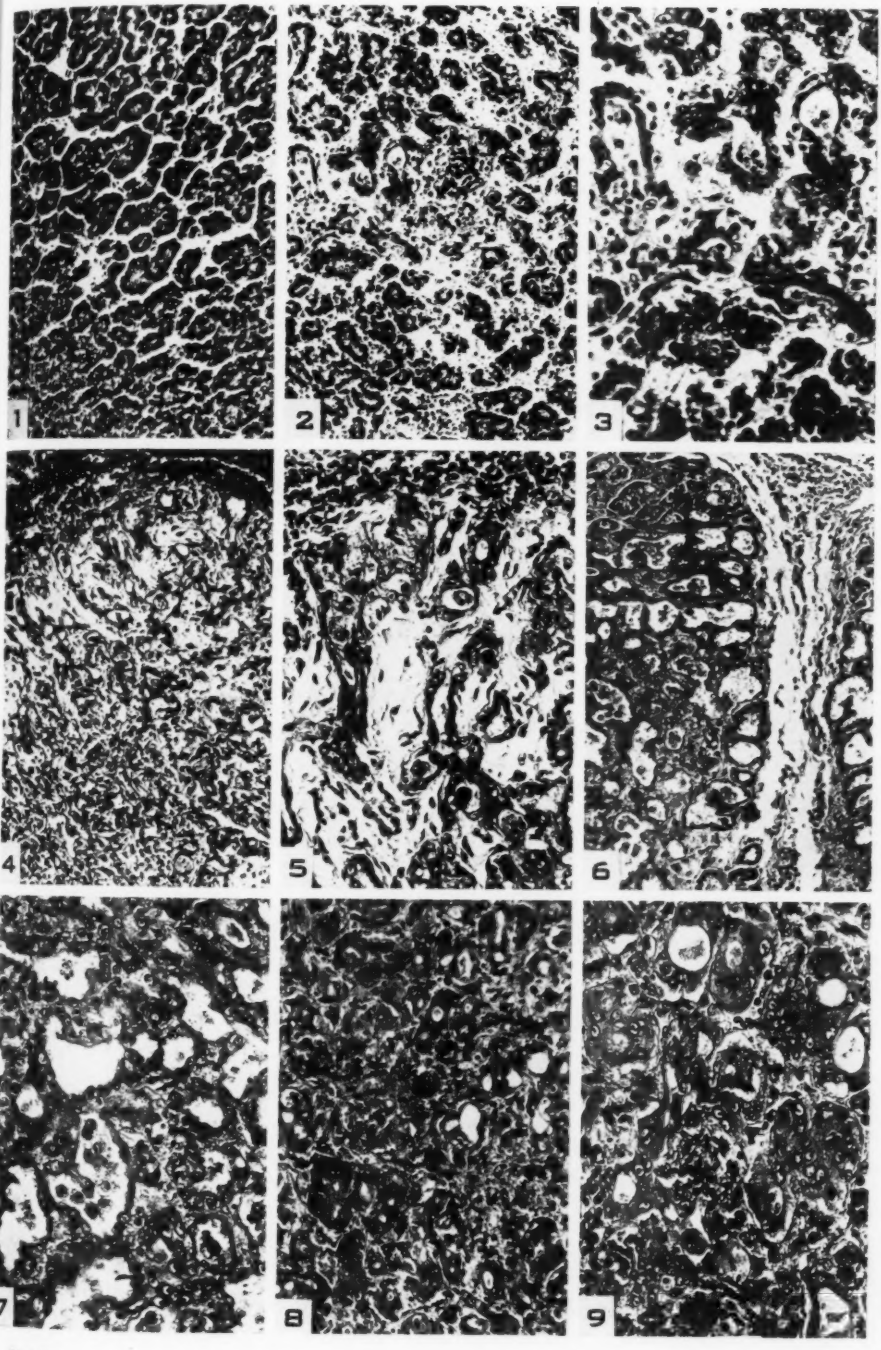
FIG. 5. The outer margin of a second stage lesion adjacent to that shown in Figure 4, showing delicate branching tubules which extend to the margin of splenic tissue. × 200.

FIG. 6. Third stage lesion of the pancreas of a timber rattlesnake (*Crotalus horridus*). Two adenomatous lesions may be seen, separated by fibrous tissue which passes in from the capsule. × 90.

FIG. 7. Left central part of tissue shown in Figure 6. × 200.

FIG. 8. Fourth stage lesion of the pancreas of a black racer (*Coluber constrictor*). This field is representative of the entire organ. No trace of acini or islet cells was seen. × 90.

FIG. 9. Cellular character of a part of the field shown in Figure 8. × 200.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